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THE ROLE OF INFECTION IN RADIATION INJURY

C. PHILLIP MILLER, M.D., CAROLYN W. HAMMOND, PH.D., AND MARIANNE TOMPKINS, B.S.
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THIS investigation was undertaken in an effort to estimate the importance of infection as a factor in the death of animals exposed to ionizing radiation. It is a sequel of radiation injury which has received less attention than it deserves, possibly because the most familiar criterion of infection is lacking. Accumulation of pus cannot occur because the necessary cellular elements have been destroyed. The literature has been reviewed by Warren and Dunlap.[1] Most of it can be divided into two categories: (a) pathologic observations on autopsy material, and (b) experimental studies, which included bacteriologic observations.

The references in the first category contain little more than passing mention of the presence of bacteria in microscopic preparations of blood or tissues, such as those of the victims dying several days after exposure to the atomic bomb at Hiroshima or Nagasaki[2-9] and of animals[10-12] used in the tests at Bikini. These observations were not confirmed by culture because bacteriologic facilities were lacking.

In the second category are a few experimental studies made on laboratory animals.[13-18] Warren and Whipple,[13] in their experiments on x-ray intoxication, obtained negative blood cultures from three dogs which were killed one, two, and three days after exposure to a lethal dose of x-ray. Two dogs killed on the fourth day both had positive blood cultures.

Mottram and Kingsbury[14] found that rats and guinea pigs developed bacteremia after irradiation with x-ray or radium. Although their experiments seem to indicate that irradiation lowered the animals' resistance to infection, the authors concluded that the bacteremia resulted from damaged intestinal epithelium rather than lowered resistance.

In a more extensive investigation, Chrom[15] cultured the blood and organs of mice killed at daily intervals after irradiation with 550 r. All of ten mice had negative blood cultures from the third to sixth days, but four of seven mice had positive cultures from the seventh to tenth days.

Lawrence and Tennant[16] found that blood and viscera were sterile in mice killed one, two, three, or four days after x-radiation with 1,000 r (LD_{50} = 550 – 600 r.). A few which died (spontaneously) during the first week had positive cultures, as did most of those which died five to eleven days after irradia-

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tion with 700 to 800 r. The authors concluded that "the mechanism of death after . . . irradiation seems to be a combination of tissue destruction or enterogenous infection; the former predominating in the acute deaths after large doses."

Bennett, Rekers, Kresge, and Howland[17] made blood cultures within twenty-four hours post mortem on twenty-two of thirty-three dogs which died after exposure to midlethal doses of x-ray. Of these, seventeen (77 per cent) had positive blood cultures.

METHODS AND MATERIALS

Mice.—The animals used in these experiments were male white mice obtained from a single breeder.* They averaged 20 grams. The mice were shipped to the laboratory by express and were in transit overnight. They were held in quarantine from four to eleven days before they were used.

The animals were kept in a constant temperature room maintained at 73° to 78° F. The humidity was not controlled.

They were housed either in (a) boxes made of sheet metal and wire netting and measuring 8 by 12 by 9 inches, the floors of which were covered with wood shavings; or (b) in stainless steel cages measuring 15 by 9 by 7 inches with wire screen floors through which the feces dropped into a pan below. The numbers confined in the larger cages never exceeded forty, nor in the smaller cages, twenty.

In the beginning of this study, the mice were fed a diet of whole wheat bread and water. This was soon changed to Purina Dog Checkers and later to mice pellets (Derwood), made according to a formula prescribed by the Argonne National Laboratory. The water bottles were changed daily.

Methods of Irradiation.—The mice were subjected to 600 or 450 r. total body irradiation in a single exposure, delivered at 250 kv., 15 Ma., at a distance of 27 inches using ½ mm. copper and 3 mm. bakelite filters, at a rate of approximately 20 r. per minute. Sixteen mice at a time were placed in plastic tubes measuring 3 cm. in diameter and 10 cm. in length, perforated to allow adequate ventilation. The tubes were closed with cork stoppers and fastened by elastic bands to a circular plate which rotated slowly within the irradiated field. Most of the mice were irradiated at the Argonne National Laboratory with the assistance of Mr. Joseph Trier and Mr. Emil Johnson.† A few of the mice were irradiated at the Billings Hospital by Dr. James W. J. Carpender, Assistant Professor of Radiology, University of Chicago.

Five hundred eighty-three mice were irradiated with 600 r. and 1,035 with 450 r.

The LD_{50} for these mice was determined by subjecting seven groups of sixteen each to total body irradiation at doses ranging from 300 to 600 r. (in increments of fifty). The LD_{50} computed by the Reed-Muench formula was 393 r.

Autopsy Cultures.—The mice were etherized and autopsied under aseptic precautions. Blood was drawn from the heart into a capillary pipette and planted immediately onto the media described below. The spleen was removed, placed in a sterile Petri dish, and cut in half for culture.

All viscera were carefully inspected, and a note made of any gross pathologic change. No microscopic examinations were carried out.

Certain changes in the culture procedure were made after the 600 r. series had been completed. The methods used in the two series must therefore be described separately.

*Maple Grove Rabbitry, Springfield, Mo. The breeding stock was started in 1909 from twenty Swiss mice to which no additions from outside sources have been made. The strain had been used in this laboratory regularly for many years.

†We are indebted for this service to Dr. Austin Brues, Director of the Division of Biological and Medical Research, Argonne National Laboratory.

600 r. Series.—A drop (0.04 to 0.05 ml.) of heart's blood from the capillary pipette was spread on the surface of a nutrient agar plate and another drop on a blood agar plate (5 per cent defibrinated sheep's blood in nutrient agar*).

The cut surface of one piece of the spleen was streaked onto nutrient and blood agar plates and the other piece dropped into 5 ml. of nutrient broth.

All cultures were incubated at 37° C. for twenty-four hours and examined. Negative cultures were incubated for an additional twenty-four hours and re-examined. Colonies appearing on the agar plates were smeared and stained by Gram's method. Positive (spleen) broth cultures were streaked onto both eosin-methylene-blue and blood agar plates. The gross appearance of the colonies developing on these plates was compared with that of the colonies found on the heart's blood plates. Smears were made and stained with Gram. In no instance were colonies found on the heart's blood plates that did not appear on the spleen broth plates. In a few cases the spleen broth was positive while the heart's blood plates were negative. Therefore, isolated colonies were picked from spleen broth plates and inoculated into nutrient broth. After twenty-four hours' incubation these broths were streaked onto nutrient agar to make sure that the cultures were pure. One colony was picked and planted onto an agar slant which after twenty-four hours' incubation was placed in the refrigerator until the organism could be identified.

Anaerobic cultures were made of the heart's blood of twenty-five of the mice in this series using Brewer's thioglycollate medium.

450 r. Series.—As this series was begun after the 600 r. series had been completed, a few modifications in method were made as a result of experience gained previously.

As much blood as possible was obtained from the heart and, after two drops had been used to inoculate the two plates (nutrient and blood agar), the remainder was put into a tube of brain heart infusion broth.

This broth was substituted for nutrient broth because it was more favorable for the growth of alpha hemolytic streptococci, which had been encountered in the 600 r. series.

The spleen was not cultured directly onto plates because in the 600 r. series such cultures were less frequently positive than those in liquid media. In a majority of the animals a large portion of the spleen was removed and dropped into a tube of brain heart infusion broth which was incubated aerobically.

In sixty animals it was cut in half and each portion dropped into a tube of broth to be incubated aerobically and anaerobically. Anaerobic conditions were secured by means of sodium hydroxide and pyrogallic acid, and the tube was sealed with a paraffined cork. These broths were incubated four days and then were streaked onto blood agar plates. These plates were incubated in a Brewer's jar using illuminating gas to replace evacuated air. After four days' incubation, the jars were opened and the plates examined.

Identification of Microorganisms.—All the Gram-negative rods were identified in the following manner: The twenty-four hour growth on an agar slant was used to inoculate the following carbohydrate broths in tubes containing gas traps: lactose, dextrose, maltose, mannite, sucrose, and xylose. In addition to these sugar media, Koser's citrate, urea, gelatine, lead acetate, and Difco's motility media were employed. Voges-Prauskauer and methyl red tests were made, and indole production was determined.

No attempt was made to identify the alpha hemolytic streptococci other than to test their ability to grow at 45° C.

The anaerobes were not identified specifically. Their morphology was noted, as well as their ability to produce hemolysis.

Control Cultures on Normal Mice.—Blood and spleen cultures were made on thirty-five normal mice from the same stock, using the methods described above for the 450 r. series. All of these cultures remained sterile.

In a further attempt to rule out the presence of latent infection in our stock mice, an additional seventeen were killed and autopsied. The spleen of each was removed,

*Difco.

ground separately in a Waring blendor, and cultured aerobically and anaerobically. All of these cultures remained sterile.

The bacterial flora of the gastrointestinal tract at different levels was examined by the following method which was essentially that described by Webster.[19] Ten normal mice were killed with ether, and the abdomen opened aseptically. The entire intestine was removed and placed in a sterile Petri dish. Sections approximately 1 cm. in length were removed from the duodenum, ileum, cecum, and midportion of the colon. Each was slit lengthwise and placed in test tubes containing 5 ml. of sterile saline and a few glass beads.* An attempt was made to select sections containing equal amounts of feces. Since this was not always possible, the results were not strictly quantitative. They did, nevertheless, provide the desired information concerning the bacterial flora of the several portions of the gut of these mice.

After thorough mixing in the test tubes, dilutions were made as follows:

Duodenum	1:20
Ileum	1:50
Cecum	1:500 and 1:5000
Colon	1:50 and 1:500

One-tenth milliliter of each dilution was dropped onto blood agar, E. M. B., and 0.5 per cent dextrose agar plates. The inoculum was spread by means of glass beads. The plates were incubated twenty-four hours at 37° C. and were read. When two dilutions were used, the one showing the best distribution of colonies was read.

The results presented in Table I show that of the four regions cultured, the duodenum contained the fewest bacteria. The ileum contained somewhat more, and the large bowel still more, both in number and in variety. The flora of the two regions of the large bowel were practically identical in kind, but that of the cecum consisted of approximately ten times as many microorganisms as that of the midportion of the colon. Of the few microorganisms in the duodenum, alpha hemolytic streptococci were relatively more frequent than in the lower portion of the bowel.

It should be noted that Pseudomonas was not encountered among these cultures, but was found later in cultures of feces of normal mice.

RESULTS

Mice Irradiated with 600 r.—In this series 288 mice were killed and cultured, twenty for each postirradiation day from the second through the fifteenth day (plus an extra mouse on the fifteenth day). To obtain this number of cultures on sacrificed animals, it was necessary to irradiate five groups of mice, a total of 583, since 295 of them died during the course of the experiments.

No cultures obtained from mice which died are included in the data for the following reasons: (a) preliminary experiments showed that the majority of blood cultures of mice which had died (spontaneously) were positive; (b) it was important to avoid the possibility of post-mortem contamination which might introduce a complicating factor into the results; and (c) our primary objective was to determine the role of infection as a *cause* of death.

The results are presented in detail in Table II, which shows the number of mice from which positive cultures were obtained, the number of mice cultured, and the number which died on each postirradiation day in each group. Mice with positive spleen and negative blood cultures are indicated by the parentheses enclosing a figure. In no instance was a mouse found to have a positive blood and negative spleen culture.

*Glass balls (about 6 mm. in diameter) customarily used for defibrinating blood. See Miller and Bohnhoff.[20]

TABLE I. BACTERIAL FLORA OF INTESTINAL TRACT OF CONTROL MICE

MOUSE NO.	ORGANISM	DUODENUM 1:20*	ILEUM 1:50	CECUM 1:5000	COLON 1:500
1	Coliform	–	+†	++++	++++
	Alcaligenes	–	+	+	+
	Lactobacilli	–	++	++++	++++
	α strep	+	++	++++	++++
	Others	–	–	+	+
2	Coliform	–	–	+++	+++
	Lactobacilli	–	++	++++	++++
	α strep	–	++	++++	++++
	Others	–	+	+	+
3	Coliform	–	+	+++	+++
	Proteus	+	++	+++	+++
	Alcaligenes	–	–	+	+
	Lactobacilli	–	++	++++	++++
	α strep	+	++	+++	+++
	Others	–	+	+	+
4	Coliform	+	++	+++	+++
	Paracoli	–	–	++	++
	Proteus	+	+	++	++
	Alcaligenes	–	–	+	+
	Lactobacilli	+	++	+++	+++
	α strep	+	++	+++	+++
	Others	–	+	+	+
5	Coliform	+	++	+++	+++
	Paracoli	–	+	++	++
	Lactobacilli	–	–	++	++
	α strep	–	–	++	++
	Others	–	–	+	+
6	Coliform	–	+	++	++
	Alcaligenes	–	–	++	++
	Lactobacilli	–	+	+++	+++
	α strep	+	+	+++	+++
	Others	+	+	+	+
7	Paracoli	–	+	++	++
	Alcaligenes	–	–	+	+
	Lactobacilli	–	++	+++	+++
	α strep	+	++	+++	+++
	Anaerobes	–	–	–	+
	Others	+	+	+	+
8	Coliform	–	+	+	+
	Alcaligenes	+	+	+	+
	Lactobacilli	–	++	+++	+++
	α strep	–	+	–	–
	Anaerobes	–	–	–	+
	Others	–	+	+	+
9	Coliform	–	–	+	+
	Proteus	–	+	++	++
	Lactobacilli	–	++	++++	++++
	α strep	++	++	++++	++++
	Others	+	+	+	+
10	Coliform	–	+	+++	+++
	Paracoli	–	+	++	++
	Lactobacilli	–	+	+++	+++
	Others	+	+	+++	+++
	α strep	–	+	+	+

*Dilution of suspension described in text.

†Number of colonies developing from 0.1 milliliter.

– = no growth
\+ = <50 colonies
++ = 50-100 colonies
+++ = 100-200 colonies
++++ = >200 colonies

Pseudomonas was not encountered among these cultures, but was found later in cultures of normal mice.

TABLE II. DAILY RECORD OF POSITIVE

DATE OF IRRADIATION	NO. MICE	DAYS POSTIRRADIATION							
		2	3	4	5	6	7	8	9
12-14-48	117	1/5*	1/5	0/5	3 D 0/5	1 D 2/5	3 D 3/5	6 D 2/5	14 D 4/5
12-29-48	119		1 D	2 D	5 D 0/5	6 D 3/5	12 D 5/5	15 D 2(1)/5	12 D 3/5
1-4-49	118		1 D 0/5	1/5	3 D	8 D 4/5	2 D 1/5	5 D 5/5	12 D 5/5
1-18-49	120		0/5	0/5	1 D 0/5	7 D 0/5	2 D 2/5	7 D 1/5	7 D 5/5
2-21-49	109	0/15	0/5	1 D 0/5	2 D 1/5	1 D	3 D	2 D	7 D
Total Mice	583								
No. Killed and Cultured		20	20	20	20	20	20	20	20
% Positive		5.0	5.0	5.0	5.0	45.0	55.0	50.0	85.0
Deaths		0	2	3	14	23	22	35	52
% Mortality†		0.34		3.1		9.2		22.0	

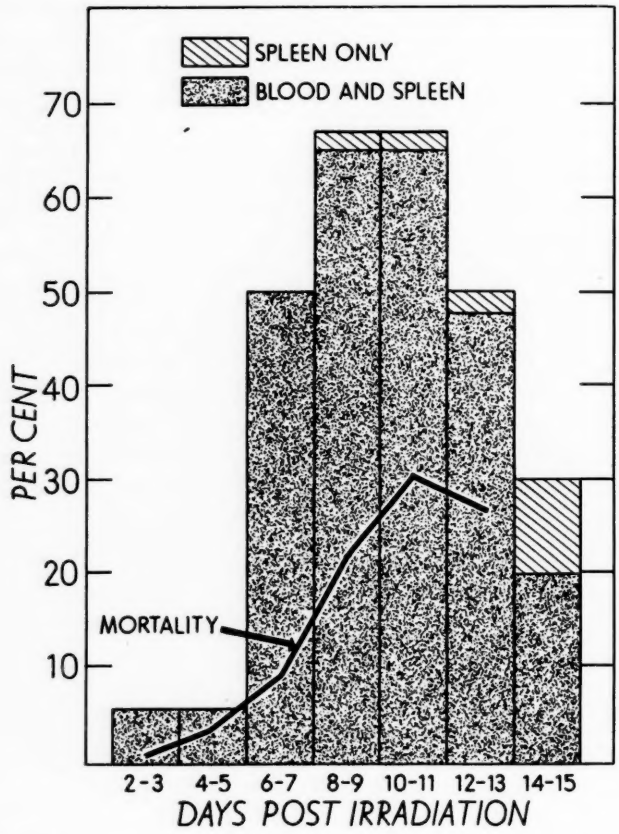

Fig. 1.—Incidence of positive cultures and mortality after irradiation with 600 r.

CULTURES AND OF DEATHS (600 R.)

DAYS POSTIRRADIATION							TOTALS	
10	11	12	13	14	15	16	KILLED & CULTURED	DIED
13 D 4/5	17 D	4 D	4 D 2/5	1 D 0/1			51	66
5 D 2/5	5 D	5 D 3/5	4 D 3/5	1 D 0/5	0/1		46	73
9 D 3/5	7 D 5/5	8 D 2/5	2 D 1(1)/5	3 D	3 D 2(2)/5		55	63
10 D 3/5	8 D 3/5	4 D 2/5	4 D 3/5	2 D 2/5	1 D 3(2)/5	0/2	67	53
5 D	5 D 7(1)/10	1 D 4/5	5 D	8 D 4/9	1/10	3(3)/5	69	40
							583	
20	20	20	20	20	21	7		
60.0	75.0	55.0	45.0	30.0	30.0	42.0		
42	42	22	19	15	4	0		
30.8		27.7		28.3				

*Denominator = number of mice killed and cultured. Numerator = number of mice yielding positive spleen and/or blood cultures. ( ) = number of these mice yielding positive spleen cultures only. D = died, not cultured.

†Mortality based on the number of survivors alive at the beginning of each two-day period.

The material contained in Table II has been summarized in Fig. 1. The columns represent the percentage of cultures which were positive. Two days' results have been combined in order to increase to forty the number of mice on which each percentage was computed. The height of each column, therefore, indicates the percentage of mice showing positive cultures in each two-day period.

The mortality curve shows the rate at which "spontaneous" deaths occurred. Each point represents the "spontaneous" deaths occurring during each two-day period, plotted as per cent of the survivors alive at the beginning of that period. The last point is omitted because too few mice remained.

As shown in Table II and Fig. 1, positive cultures, with few exceptions, were cultures of heart's blood. These indicate a high incidence of bacteremia during the second week postirradiation, particularly during the four-day period from the eighth to the twelfth day when the mortality was greatest. The severity of the bacteremia will be discussed below.

Mice Irradiated with 450 r.—The results in this series are presented in detail in Table III and summarized in graphic form in Fig. 2. In this series, groups of mice totaling 1,035 were irradiated. Thirty-five mice were killed and cultured on each day postirradiation—a total of 597. The columns in Fig. 2 indicate the percentage of positive cultures among the seventy mice which were killed in each two-day period. It will be seen that the incidence of bacteremia was lower in this series of mice than in the one irradiated with 600 r., and also that a considerable proportion of the animals had positive spleen and negative blood cultures.

TABLE III. DAILY RECORD OF POSITIVE

DATE OF IRRADIATION	NO. MICE	DAYS POSTIRRADIATION									
		1	2	3	4	5	6	7	8	9	10
12-7-48	98		0/5*	0/5	1 D 0/6	1 D 0/5	2 D 1/5	1 D 0/5	1 D 1/5	3 D 1/5	1 D 3/5
5-16-49	185		2(2)/10	0/10	4(4)/10	4 D 1(1)/10	3 D 2/10	5 D 2(1)/10	5 D 1/10	1(1)/10	11 D
6-6-49	135		0/5	0/5	0/5	5 D 1(1)/5	1 D 0/5	12 D 0/5	5 D 1/5	10 D 0/5	4 D 8(3)/15
6-13-49	137		1/5	0/5		1 D	7 D	12 D	14 D 2(1)/5	10 D 3/5	16 D 2(1)/5
6-20-49	18					1 D	2 D			4 D	4 D
6-27-49	181	1 D	0/5	0/10	2(1)/10	2 D 1(1)/10	6 D	11 D	20 D 4/10	6 D 1/10	8 D 6/10
7-11-49	106					1 D	2 D	6 D	9 D	11 D	21 D
7-18-49	39	5 D			2 D	2 D	3 D	5 D	6 D	5 D	2 D
8-13-49	2†										
9-6-49	15†		0/2			2 D	2 D	2 D	3 D	2 D	
9-7-49	2†		0/2								
9-12-49	20								1 D	1 D	
9-13-49	20		0/1			1 D	1 D 2/15	0/2			
9-14-49	20							1 D 2/13			
9-15-49	20					0/5					2 D
9-19-49	20				0/4		1 D		1 D	1 D	1 D
9-22-49	20						1 D			1 D	
Total Mice	1035										
No. Killed and Cultured		0	35	35	35	35	35	35	35	35	35
% Positive		0	8	0	17	8	14	11	25	17	54
Deaths		6	0	0	3	20	31	55	65	54	70
% Mortality‡			0		2.3			9.9		16.7	

*Denominator = number of mice killed and cultured. Numerator = number of mice yielding positive spleen and/or blood cultures. ( ) = number of these mice yielding positive spleen cultures only. D = died, not cultured.

†Taken from a group of twenty mice irradiated for another purpose.

‡Mortality based on the number of survivors alive at the beginning of each two-day period.

Bacteria Recovered from Blood and Spleen Cultures.—The microorganisms recovered from the cultures of blood and spleen are listed in Table IV, according to the genera to which they belong. Comparison with Table I shows that all of them were members of the normal intestinal flora of our stock mice.

Most of the positive cultures were pure cultures. Ninety-one per cent of them consisted of a single species. The remaining 9 per cent contained only two microorganisms.

CULTURES AND OF DEATHS (450 r.)

DAYS POSTIRRADIATION										TOTALS	
11	12	13	14	15	16	17	18	19	20	KILLED AND CULTURED	DIED
0/5	1 D 1/5	1 D 0/5	1 D 0/5	0/5	1/5	1 D 1/5			1 D	81	15
4 D 6(1)/10	1 D 7(1)/10	1 D	5 D	5 D 5/10	3/10	0/10	5(1)/11			141	44
	9 D	2 D		4(2)/5	2 D 4(1)/5	1 D 1/5	0/10	1/4		84	51
4 D 4/5	3 D 3(1)/5	7 D 4(1)/5	5 D	1/5	1 D 3/5	1/5	0/2			57	80
3 D 3(1)/4										4	14
7 D 3/10	7 D 4/10	9 D 9/14	2 D 0/3							102	79
11 D 1/1	8 D 2/5	6 D 2(1)/6	4 D 11(1)/15							27	79
4 D		1 D	1 D 2/2				1 D			2	37
		0/2								2	0
	1 D		1 D							2	13
										2	0
	1 D		1 D	1(1)/10	2(2)/6					16	4
										18	2
		1(1)/3				0/3				19	1
			1 D 0/10				0/2			17	3
							1(1)/10	1 D		14	5
	2 D	2 D		1 D	0/4	0/7	0/2			13	7
										1035	
35	35	35	35	35	35	35	37	4			
48	48	45	37	31	37	8.5	16	25			
33	33	29	21	6	3	2	1	1	1		
19.7	17.8		12.5		4.1						

TABLE IV. CLASSIFICATION OF MICROORGANISMS RECOVERED FROM HEART'S BLOOD AND/OR SPLEEN

Paracolobactrum	42 %
Coliform	22 %
Proteus	13 %
Pseudomonas	9 %
Alpha Streptococcus	6 %
Unidentified gram-negative rod	3 %
Alcaligenes	2 %
Anaerobes	0.3 %

TABLE V. SEVERITY OF THE BACTEREMIA—AS INDICATED BY COLONY COUNTS ON POSITIVE BLOOD CULTURES

COLONIES PER DROP* OF BLOOD	PER CENT OF ALL POSITIVE CULTURES	
	IN 600 r. SERIES	IN 450 r. SERIES
<50 Colonies	70%	78%
Innumerable colonies	36%	18%

*From a capillary pipette equal to approximately 0.04 to 0.05 milliliter.

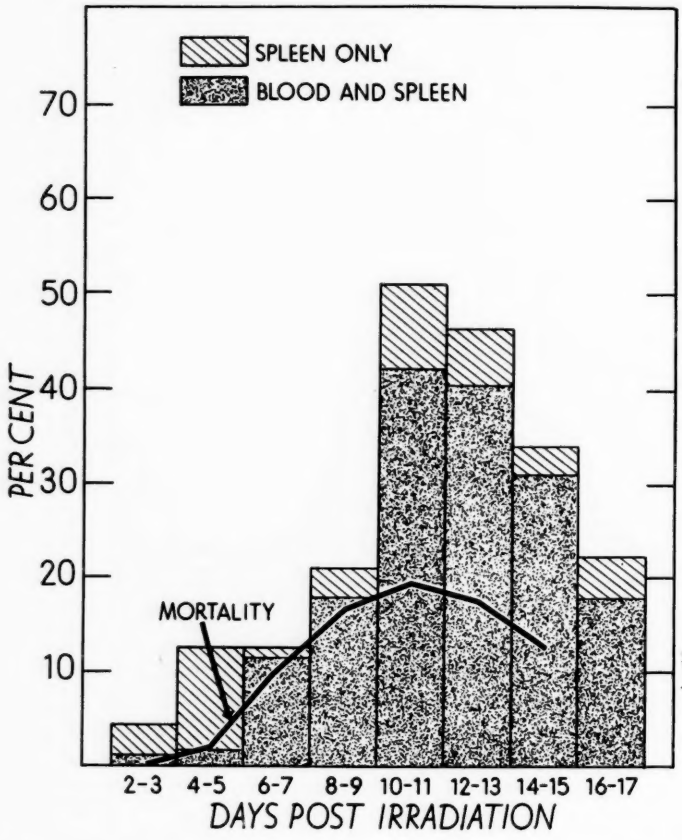

Fig. 2.—Incidence of positive cultures and mortality after irradiation with 450 r.

Severity of Bacteremia.—The severity of the bacteremia is illustrated in Table V.

Thirty-six per cent of the mice having septicemias in the 600 r. series were infected to such a degree that innumerable colonies developed from a single drop of heart's blood. Seventy per cent of the bacteremic mice in this group showed more than fifty colonies per drop of heart's blood.

In the 450 r. series 18 per cent of the positive heart's blood cultures were found to contain innumerable colonies per drop, while 78 per cent yielded more than fifty colonies.

Gross Pathologic Findings.—The most frequent abnormality observed at autopsy was hyperemia of the cecum, particularly in the 600 r. series. The next

most common findings were anemia and hemorrhage. Hemorrhage, which was always limited to small areas, was found most frequently in the subcutaneous tissue, but was occasionally seen in the cecum and less frequently in other portions of the gastrointestinal tract.

DISCUSSION

These experiments demonstrate that bacteremia occurs in mice subjected to 600 or 450 r. total body x-radiation during the second week after exposure—the period of greatest mortality. This observation is in agreement with the earlier findings of Chrom[15] and of Lawrence and Tennant[16] on mice and of Warren and Whipple[13] and Bennett, Rekers, Kresge, and Howland[17] on dogs.

Our findings were based on cultures of blood and spleen, whereas the cultures of Chrom and of Warren and Whipple included several other organs. Our observations, however, were made on a much larger series of animals.

It should also be pointed out that our experiments had as their single purpose the evaluation of the role of infection as a cause of death in radiation injury. They were undertaken in the hope of answering the question raised by previous workers: whether such infection occurs merely as a terminal event in an animal already dying of radiation injury, or whether infection once initiated leads inevitably to death.

Our results seem to support the latter alternative for the following reasons: (1) In our experiments the possibility of post-mortem contamination was eliminated and the probability of agonal infection of the blood stream was minimized by culturing only *killed* mice. The high incidence of bacteremia found in killed mice makes it clear that infection is a common occurrence during the second week after irradiation with 450 r., and occurs even more frequently among those irradiated with 600 r. (2) The bacteremia was severe. In approximately three-fourths of the positive blood cultures the colony counts exceeded fifty per drop of blood. (3) The bacteremia in most instances was caused by a single microorganism, which being found in such large numbers suggests that active multiplication must have been occurring in the blood stream. In other words, most of the mice were suffering from an overwhelming sepsis at the time they were killed.

These observations support the conclusion that infection was of such a degree as to be incompatible with life. The decisive test would be made in experiments in which bacteremia had been prevented. Such experiments have in fact been made and have shown that the administration of suitable antibiotics reduced the mortality of mice irradiated with 450 r. to a significant degree.[21] The conclusion seems justified, therefore, that infection plays an important role as a cause of death in mice irradiated with moderate doses of x-ray.

The source of infection in these mice was clearly the lower gastrointestinal tract. All of the microorganisms recovered from the blood or spleen were members of the normal flora of the large bowel. It is of interest that the cecum, the region in which such bacteria were most numerous, was the region in which gross pathology, particularly hyperemia, was most frequently observed in the irradiated mice.

It seems probable, therefore, that the bacteria in the lower bowel gained entrance to the blood stream and multiplied there unrestrained by the animal's defense mechanism which had been rendered ineffective by the ionizing radiation. As a result, a septicemia developed which led to death.

It must not be concluded, however, that these facts provide a complete explanation for the occurrence of infection in irradiated animals. Investigation of the sequence of events which precede and presumably result in the development of bacteremia has been undertaken.

SUMMARY

Male white mice averaging 20 Gm. in weight were subjected to 600 or 450 r. total body x-radiation in a single exposure.

In the 600 r. series, 288 mice were killed and cultured (at the rate of twenty a day). Of these, 113 showed positive cultures from heart's blood and/or spleen. Eighty per cent of the positive cultures occurred during the second week postirradiation, the period of greatest mortality.

In the 450 r. series 595 mice were killed and cultured (at the rate of thirty-five a day). One hundred fifty-two showed positive cultures from heart's blood and/or spleen. Sixty-six per cent of the positive cultures occurred during the second week postirradiation.

In a majority of the animals the bacteremia was severe enough to be regarded as an overwhelming sepsis. The bacteremia was caused by microorganisms normally present in the lower bowel of this strain of mice. Control cultures on unirradiated mice were all sterile.

It is concluded that septicemia caused by microorganisms of enteric origin is in large measure responsible for the death in mice irradiated with these doses of x-ray.

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ACTH IN EXPERIMENTAL POLIOMYELITIS IN MONKEYS AND MICE*

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THE present report deals with experiments carried out in order to determine whether the administration of ACTH would influence the course of experimental poliomyelitis in mice and monkeys. In addition, observations were made on the blood picture of normal monkeys and of monkeys treated with ACTH with and without infection by the virus of poliomyelitis.

MATERIALS AND METHODS

Animals.—Thirty-eight healthy, immature, Macaca mulatta monkeys which weighed 2.3 to 3.8 kg. and nineteen healthy young cynomolgus monkeys which weighed 1.3 to 2.5 kg. were used. Equal numbers of the sexes were used in each group in the two experiments with rhesus monkeys. The mice used were white Swiss, three to five weeks old and 12 to 16 grams in weight. Mice were examined daily, the day of onset and character of paralysis and day of death were recorded. Survivors were discarded after thirty days.

Virus.—(1) The Minnesota strain of poliomyelitis virus, isolated in this laboratory in 1945, was used in rhesus monkeys for the tests with ACTH. The suspension used, Minnesota Pool II, represents the fourth passage in monkeys. The spinal cords and medullae of ten rhesus monkeys, obtained within twenty-four hours of the onset of paralysis, were ground in a Waring blendor with sufficient 0.15M NaCl to make a 20 per cent suspension. The suspension was centrifuged at 1,000 r.p.m. for fifteen minutes, the coarse sediment discarded, and the supernate sealed in glass ampules in 1 to 5 ml. aliquots which were stored at approximately −70° C. in an ice chest refrigerated with solid CO_2. Shortly before use an ampule was thawed at 37° C., and diluted with 0.15M NaCl. The PD_{50} (50 per cent paralytic end point)[1] titer of Minnesota Pool II was $10^{-3.2}$ when five monkeys per log dilution were inoculated intracerebrally. In the present report, intracerebral inoculation consisted in injecting 0.5 ml. of the desired dilution of virus suspension into the region of the thalamus of a monkey anesthetized with ether.

(2) For inoculation of cynomolgus monkeys the Mahoney strain of virus isolated in this laboratory in 1941 was used. The suspension was prepared and stored as described for the

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*At the time this work was instituted, information was received through Dr. H. M. Weaver of the National Foundation for Infantile Paralysis of resul's of an experiment conducted by investigators at the Children's Hospital, Philadelphia, Pa. They had sent the data to him to make them available to a laboratory such as ours which was engaged in extensive studies of poliomyelitis in monkeys. Their data which agree in substance with ours are presented in a paper on p. 359 of this number of the J. LAB. & CLIN. MED.

Minnesota strain, except that the cords and medullae from seven cynomolgus monkeys inoculated subcutaneously were used. The subcutaneous PD_{50} titer of the Mahoney pool in cynomolgus monkeys was approximately $10^{-2.7.2}$ One milliliter of the desired dilution of virus suspension was used for each subcutaneous inoculation.

(3) Autopsies were performed on the animals at the time of paralysis, or if not paralyzed, within forty-five days after inoculation of virus. The central nervous system of each monkey was examined histologically to establish the diagnosis of poliomyelitis.

(4) In mice, two pools, M_{82} and M_{84}, of the Lansing strain of poliomyelitis virus were used. Six titrations of M_{82}, each with twenty mice per log dilution, averaged $10^{-4.06}$ (range $10^{-3.70}$ to $10^{-4.30}$). Eight titrations of M_{84}, five with twenty mice, and three with 8 or 10 per log dilution, averaged $10^{-4.55}$ (range $10^{-4.33}$ to -4.72). All mice were examined daily, and the survivors discarded after thirty days. The pools were prepared with brains and cords of mice paralyzed before the sixth day after inoculation of a 10 per cent suspension of the preceding passage. A 20 per cent suspension in 0.15M NaCl was prepared by grinding with a pestle in a mortar in the presence of alundum, and centrifuging at 3,000 r.p.m. for sixty minutes at 4° C. Small aliquots of the supernate, in rubber-stoppered tubes (M_{82}) or sealed glass ampules (M_{84}), were stored at −70° C. Mice inoculated intracerebrally received 0.03 ml. of the desired dilutions of virus suspension.

ACTH.—Three lots of ACTH were used.* The two experiments in monkeys were carried out with Lots No. 83-ASI or No. 128-105R, both 160 per cent of La-1-A standard,[3] while Lot No. 71-2(50) c (20 per cent of La-1-A standard) was used in mice and in one monkey whose urine was tested for the content of 17-ketosteroids. In the present report the dose of drug used will be referred to in terms of the equivalent of La-1-A standard ACTH. For injection into monkeys, the powder was dissolved in sufficient 0.15M NaCl to yield a concentration of 250 mg. per milliliter. For injection into mice a concentration was used of either 0.67 mg. per milliliter or 3.3 mg. per milliliter. Only that amount of drug needed for the day was dissolved, and both the powder and solution were kept at 4° C.

Cortisone.—Lot No. 9R9512 of Cortisone Acetate (Merck), supplied in a concentration of 5 mg. per milliliter was used in mice. It was stored at 4° C. and diluted 1:10 with 0.15M NaCl just before use, or used undiluted.

Clinical Laboratory Tests.—(1) The glucose tolerance tests were performed as follows: samples of blood were obtained in oxalated tubes from the femoral vessels before and one, two, and three hours after the administration by stomach tube of 3 Gm. per kilogram of glucose as a 20 per cent solution in water. The determination of glucose was carried out immediately using the Nelson[4] modification of the Somogyi photometric method.†

(2) The *hematocrit, hemoglobin* concentration, *white blood cell* count, *red blood cell* count, and *differential* counts from blood smears were determined in the usual manner. A modified Randolph[5] technique was used to make the *total eosinophil* counts. They were carried out in the manner usual for total white blood cell counts except that the diluent was 0.05 per cent phloxine in 50 per cent propylene glycol, the entire ruled areas (0.9 $mm.^3$ each) on both sides of the counting chamber were counted, and counts were completed within twenty to ninety minutes of diluting the blood in the phloxine solution.

EXPERIMENTAL

Experiments in Mice.—In each instance mice were inoculated intracerebrally with 10 to 50 LD_{50} of the Lansing strain of poliomyelitis virus after treatment with ACTH or cortisone by the intraperitoneal route had been started.

*Provided through the kindness of the Research Division of Armour and Company, Chicago, Ill.

†We wish to thank Dr. Jerome W. Conn and Dr. S. S. Fajans for contributing the services of their laboratory for these determinations.

1. *ACTH. (Experiments A1, A2, A3).*—One and two-thirds milligrams of ACTH were given three times a day for one to five days before and two weeks after the inoculation of virus. In the first experiment seventeen mice were injected at 8 A.M., 12 A.M., and 4 P.M. every day with 0.5 ml. of a solution containing 3.33 mg. per milliliter starting on the day before virus. In the second and third experiments, 0.1 ml. of a concentration of 16.7 mg. per milliliter was used and injections were at eight-hour intervals. In the second experiment ACTH was started in twenty mice on the day before virus, and in the third experiment the drug was started in twenty-one mice five days before virus.

2. *Cortisone Acetate. (Experiments C1, C2, C3, C4).*—In the first experiment twenty mice received 0.25 mg. in 0.5 ml. once daily beginning the day before inoculation of virus. In the other experiments the drug was used in a concentration of 5 mg. per milliliter. In the second, twenty mice received 0.5 mg. per day starting the day before inoculation of virus. In the third and fourth experiments, 2.5 mg. per day of drug was started the day before virus in seventeen mice, and eight days before in twenty-four mice, respectively.

Experiments in Monkeys 1 and 2.—Duplicate experiments with ACTH were performed four months apart in rhesus monkeys, one with Lot No. 83ASI and one with Lot No. 128-105R. A dose of 25 mg. of ACTH was injected intramuscularly every six hours, beginning three days before the inoculation of virus. After the twelfth dose of ACTH the drug was continued in one-half of the treated animals at six-hour intervals until the onset of paralysis. The only exception was the thirteenth dose which was inadvertently diluted twice so that one-half was injected before and one-half after the inoculation of virus. Intracerebral inoculation with 0.5 ml. of a 2 per cent suspension of Minnesota Pool II virus (30 PD_{50}) was performed seven to eight hours after the treated monkeys had received the twelfth dose of ACTH. The drug was discontinued in six monkeys in an effort to test the effect of relative inactivity of the adrenal cortex which would be expected to follow the cessation of administration of exogenous ACTH.

In the first experiment glucose tolerance tests were performed in each of the nineteen rhesus monkeys five and ten days before, as well as three and six days after the administration of ACTH to twelve of them had been started. At the same time the hematocrit, hemoglobin content, white blood cell, red blood cell, and total eosinophil counts were determined for each monkey in addition to making differential counts from stained blood smears of each. In the second experiment only total eosinophil counts were determined on most of the eighteen rhesus monkeys three times before starting ACTH, on the third day after, and at the time of paralysis which on the average was twelve days after starting the ACTH treatment.

Experiment 3.—Nineteen cynomolgus monkeys were studied three months after the second experiment in an effort to study the effects on the eosinophil count of routine procedures of handling and inoculation. Six cynomolgus monkeys were inoculated with a 1 per cent suspension of Mahoney virus, three intracerebrally and three subcutaneously, and another six were similarly inoculated with a 1 per cent suspension of cord and medulla from a normal monkey. In addition to three uninoculated controls, four monkeys were fed 0.5 Gm. of the Mahoney virus as a 20 per cent suspension on each of two consecutive days in an unsuccessful attempt to infect them by this route. Total eosinophil counts were obtained before and at two to three day intervals for twelve days as well as on the twenty-sixth day after inoculation.

In a preliminary experiment with one immature female rhesus monkey, the excretion of 17-ketosteroids in the urine was determined[6*] before, during, and after the daily administration of ACTH. The equivalent of 100 mg. of standard ACTH (lot No. 71-2(50)c, 20 per cent of La-1-A standard) was given in three equal divided doses for seven days. Pools of the total urine excreted during periods of three to four days were tested. Hematologic measurements similar to those made in the first experiment were obtained, in addition to measuring the fasting blood sugar on eleven of fourteen days before, and six of seven days after, starting the ACTH.

*We wish to thank Dr. Jerome W. Conn and Dr. S. S. Fajans for contributing the services of their laboratory for these determinations.

RESULTS

Experiments in Mice.—(Table I) ACTH had no consistent effect upon the course of poliomyelitic infection in mice when 5 mg. were administered daily, in three equal divided doses, starting one and five days before the inoculation of virus. *Cortisone* in single daily doses of 0.25, 0.5, and 2.5 mg. similarly had

TABLE I. EFFECT OF ACTH AND CORTISONE ON INFECTION OF MICE WITH 10-50 LD_{50} OF THE LANSING STRAIN OF POLIOMYELITIS VIRUS

TEST NO.	DRUG	TREATMENT DAILY DOSE* (MG.)	STARTED BEFORE VIRUS (DAY)	RESULTS		
				MORTALITY†	INCUBATION PERIOD: 50% DEAD OR PARALYZED (DAY)	INCUBATION PERIOD: MEAN (DAYS)
A1 & C1	None			19/19	11	10.4
	ACTH	5	1	17/17	3	5.1
	Cortisone	0.25	1	20/20	7	7.4
A2 & C2	None			20/20	9	9.2
	ACTH	5	1	20/20	10	10.6
	Cortisone	0.5	1	20/20	10	11.2
A3	None			8/8	4	5.8
	ACTH	5	5	20/21	9	9.5
C3	None			20/20	11	10.1
	Cortisone	2.5	1	17/17	11	11.5
C4	None			20/20	8	10.2
	Cortisone	2.5	8	23/24	10	9.5

*ACTH given in three equally divided doses; cortisone given in a single dose.
†Number paralyzed and dead divided by number inoculated.

no consistent effect upon the infection. Although in the first experiment the animals receiving either drug exhibited a somewhat shorter incubation period, this effect was not observed in the subsequent experiments.

Experiments in Monkeys.—(Tables II to XI, Fig. 1) In the first experiment, five of twelve monkeys treated with ACTH were paralyzed by the seventh day, at which time none of the controls were yet paralyzed (Table II). In the

TABLE II. NORMAL AND TREATED RHESUS MONKEYS
PREPARALYTIC INCUBATION PERIODS, IN DAYS, AFTER INTRACEREBRAL INOCULATION OF POLIOMYELITIS VIRUS

EXPERIMENT NO.	ACTH: BEFORE AND AFTER VIRUS INOCULATION: INDIVIDUAL	ACTH: BEFORE AND AFTER VIRUS INOCULATION: AVERAGE	ACTH: BEFORE VIRUS INOCULATION: INDIVIDUAL	ACTH: BEFORE VIRUS INOCULATION: AVERAGE*	CONTROL: INDIVIDUAL	CONTROL: AVERAGE*
1	5,7,7,7,8,9	7.2	6,9,9,11,(+),†(+)†	8.8	8,9,11,11,(−),†(−)†	9.8
2	5,5,5,8,9,11	7.2	7,7,8,8,8,9	7.8	7,7,7,8,8,10	7.8
1 and 2	12 monkeys	7.2	10 monkeys	8.2	10 monkeys	8.6

*Average preparalytic period of clinically positive monkeys only.
†Result of histologic examination of CNS of unparalyzed monkey.

second experiment, three of the six treated continuously with ACTH were paralyzed on the fifth day, while the first paralysis in the other two groups occurred on the seventh day. The combined results show that four of twelve monkeys

treated continuously with ACTH were paralyzed on the fifth day, two to three days earlier than the first paralysis in the group of twelve untreated controls. Thus, the administration of ACTH resulted at the most in a slight acceleration of the onset of paralysis in three of the four treated groups with averages of 0.6, 1.0, and 2.6 days less than those of the controls (Table II). This was most suggestive in the two groups which received ACTH both before as well as after inoculation of virus. Of the four monkeys which did not develop paralysis, only the two in the untreated control group were negative for poliomyelitis upon histologic examination of the central nervous system (Table II, Experiment 1). Although the results suggested that the administration of ACTH might speed up the onset of clinical manifestations after inoculation of virus, there was no evidence in these data that the incidence or severity of infection was altered (Table II).

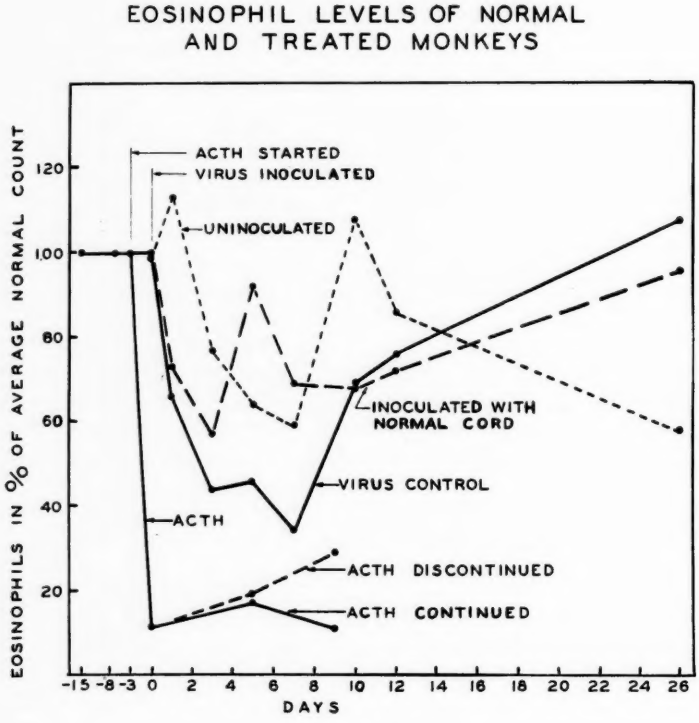

Fig. 1.

There was a marked depression of the total eosinophil count during the administration of ACTH followed by a slow rise in the count upon cessation of the drug (Table III, V, IX, Fig. 1). The eosinophil counts of monkeys inoculated with virus were also depressed below the normal range, but to a lesser extent than that occurring during the administration of ACTH. Single counts on each of nineteen normal cynomolgus monkeys averaged 608 per cubic millimeter, while the averages of three to four counts made under the same conditions on each of eighteen normal rhesus monkeys was 403 per cubic millimeter (Table

TABLE III. TOTAL EOSINOPHIL COUNTS OF NORMAL AND TREATED RHESUS MONKEYS

GROUPS AND PERIODS	MONKEYS (NO.)	DETERMINATIONS (NO.)	EOSINOPHILS			
			NO. PER MM.^{3}		PER CENT OF NORMAL VALUE (CALC. FOR EACH INDIVIDUAL)	
			GRAND MEAN	RANGE OF MEANS	MEAN	RANGE
Control Groups						
Before virus	14	39	344	22-844		
5 days after virus	6	5	99	17-309	26%	5-41%
9 days after virus	6	5	291	111-522	72%	47-129%
All monkeys before treatment						
ACTH-treated groups	38	93	305	22-844		
3 days of ACTH (before virus)	24	18	38	0-166	11%	0-66%
8 to 12 days of ACTH (5 to 9 days after virus)	12	8	21	0-67	15%	0-41%
3 days of ACTH only before virus; 5 to 9 days after virus	12	10	73	0-217	22%	0-61%

IX, Experiment 2, and Table X). In both species, however, the counts were depressed to the same general range of normal after inoculation of virus: 26 and 46 per cent of normal at five days, and 72 and 67 per cent at nine to ten days, in twelve rhesus and six cynomolgus, respectively. There were no significant differences between the eosinophil responses to intracerebral and to subcutaneous inoculation of virus in cynomolgus monkeys.

The average total white blood cell count (Tables V, VII) of twelve rhesus monkeys increased 65 per cent from an average of 14,200 per cubic millimeter to 23,400 per cubic millimeter after the administration of ACTH for three days. There then followed 35 per cent decrease from the elevated level in the ACTH-treated monkeys to 130 per cent of normal five days after the inoculation of virus. A 30 per cent decrease from the average normal level of the untreated virus controls also occurred after the inoculation of virus.

The rise in white blood cells observed after three days of treatment with ACTH was accompanied by an increase in the polymorphonuclear (PMN) differential count from 50 to 88 per cent (Tables V, VIII). On the fifth day after inoculation of virus the PMN count dropped to 69 per cent in the group in which ACTH had been discontinued, remained elevated at 83 per cent in the

TABLE IV. EXCRETION OF 17-KETOSTEROIDS IN THE URINE OF A RHESUS MONKEY TREATED WITH 100 MG. OF ACTH PER DAY

DATE	POOL (DAYS)	MG. OF 17-KETOSTEROIDS	
		TOTAL	DAILY
3/22/50	3	2.1	0.70
3/25/50	3	2.3	0.76
3/28/50	3	2.2	0.73
3/31/50	3	2.3	0.76
ACTH started			
4/3/50	4	7.9	2.00
4/7/50	3	10.0	3.33
ACTH discontinued			
4/10/50	3	5.6	1.90

TABLE V. AVERAGES OF HEMATOLOGIC DATA ON NORMAL AND TREATED RHESUS MONKEYS

			NO.	DATE AND MEAN VALUES			
		GROUP	MONKEYS	4/27/50	5/3/50	5/11/50*	5/16/50
Stained smear differential (% of 200 WBC)	PMN	Control	8	50.3	58.4		45.2
		ACTH for 3 days	6	57.4	57.0	90.2	68.6
		ACTH for 8 days	6	37.7	47.0	86.5	83.2
		Mean normal value	20	48.1	54.2		
	Lymphocytes	Control	8	42.5	38.0		44.4
		ACTH for 3 days	6	35.8	39.0	7.5	27.0
		ACTH for 8 days	6	55.8	48.0	10.5	13.4
		Mean normal value	20	45.0	41.5		
	Monocytes	Control	8	3.6	2.4		5.8
		ACTH for 6 days	6	3.6	2.0	2.0	3.1
		ACTH for 8 days	6	3.8	2.1	3.0	3.0
		Mean normal value	20	3.7	2.3		
	Eosinophils	Control	8	3.4	1.0		4.2
		ACTH for 6 days	6	2.4	1.7	0.3	1.0
		ACTH for 8 days	6	1.6	2.1	0.0	0.4
		Mean normal value	20	2.5	1.6		
	Basophils	Control	8	0.2	0.2		0.4
		ACTH for 6 days	6	0.8	0.3	0.0	0.3
		ACTH for 8 days	6	1.1	0.8	0.0	0.0
		Mean normal value	20	0.7	0.4		
	Total RBC (millions/mm.^{3})	Control	8	5.08	4.95		5.04
		ACTH for 3 days	6	5.15	5.26	4.85	5.24
		ACTH for 8 days	6	4.85	5.31	5.11	5.00
		Mean normal value	20	4.98	5.09		
	Packed cell Volume (% whole blood)	Control	8	33.8	32.5		31.1
		ACTH for 3 days	6	34.9	34.5	31.2	30.6
		ACTH for 8 days	6	33.7	33.3	31.8	29.0
		Mean normal value	20	33.8	33.3		
	Hemoglobin (gm./100 ml.)	Control	8	9.5	10.3		9.5
		ACTH for 3 days	6	10.8	10.2	9.0	10.2
		ACTH for 8 days	6	9.8	10.2	9.2	9.6
		Mean normal value	20	9.9	10.3		
	Total WBC (thousands/mm.^{3})	Control	8	19.3	18.5		13.0
		ACTH for 3 days	6	15.2	11.8	23.0	16.1
		ACTH for 8 days	6	16.3	13.4	23.8	15.9
		Mean normal value	20	16.2	15.4		
Total eosinophils per mm.^{3}	Experiment 1	Control	8	144	313		99
		ACTH for 3 days	6	71	172	2	45
		ACTH for 8 days	6	202	154	2	11
		Mean normal value	20	106	223		
				8/18	8/21	9/7	9/14†	9/22
	Experiment 2	Control	6	490	424	368	393	291
		ACTH for 3 days	6	280	329	571	65	114
		ACTH for 12 days	6	342	322	449	48	34
		Mean normal value	18	381	365	459		

*All monkeys inoculated intracerebrally with virus; ACTH started 3 days before i.c. and continued for 5 days after i.c.

†All monkeys inoculated intracerebrally with virus; ACTH started 3 days before i.c. and continued for 9 days after i.c.

group continued on ACTH, and showed no significant change in the untreated, inoculated controls. A reverse effect was noted on the percentage of lymphocytes which dropped from 45 to 9 per cent after the administration of ACTH for

three days. Five days after inoculation of virus the lymphocyte count was unchanged in the controls, rose to 27 per cent in the group in which ACTH was discontinued, and remained low in the groups in which ACTH was continued. It has long been known that a reciprocal relationship exists between neutrophils and lymphocytes during the leucocytosis which accompanies many conditions,[7] in many of which stress[8] undoubtedly plays a part.

TABLE VI. NORMAL AND TREATED RHESUS MONKEYS
TOTAL RED BLOOD CELL COUNT, HEMOGLOBIN CONTENT, AND PACKED CELL VOLUME

		4/27/50			5/3/50			5/11/50*			5/16/50		
		RBC†	HB.‡	PCV§	RBC	HB.	PCV	RBC	HB.	PCV	RBC	HB.	PCV
Virus Control	465	4.70	9.7	31.0	4.1	12.2	31.0				4.90	9.0	30.0
	466	5.40	9.8	38.0	5.6	10.2	32.0				5.30	9.9	35.0
	467	5.70	10.2	35.5	5.5	9.8	31.5				5.40	9.7	32.5
	468	5.40	10.7	33.5	5.0	10.5	32.0				4.40	8.6	28.5
	469	5.00	8.5	33.5	4.3	10.5	34.5				4.35	9.7	29.0
	482	4.25	8.2	32.0	5.2	8.3	34.0				5.90	9.9	31.5
	464	5.60	9.8	34.0	4.2	11.3	34.0						
	469a	3.35	8.5	29.0									
ACTH daily 5/8–5/11	470	5.31	10.6	36.5	5.8	10.8	33.5				5.80	9.7	31.5
	472				5.3	10.5	37.5	4.90	9.5	33.0	4.80	10.8	32.5
	473	5.84	11.2	36.0	4.7	10.0	34.5	5.60	9.4	32.0	5.90	9.3	29.0
	477	5.00	11.0	34.0	4.4	9.8	31.0				4.70	10.8	32.0
	478	4.88	11.3	35.0	4.9	10.2	34.5	4.52	8.3	31.0	5.20	10.8	29.5
	479	5.01	10.1	33.0	5.9	10.0	32.0	5.50	8.8	29.0	4.80	9.9	29.0
ACTH daily 5/8–5/16	471	5.01	9.2	33.0	5.5	9.4	34.0	4.72	9.0	31.0	4.65	8.0	29.5
	474	5.09	10.3	33.0	4.9	9.5	33.0				5.80	11.0	29.0
	475	4.32	10.2	32.0	5.5	10.3	35.0				5.10	9.8	32.0
	476	4.80	10.3	35.0	5.2	10.8	32.0	5.5	9.4	32.5	4.50	9.9	28.0
	480	5.28	10.0	36.0	5.7	10.3	34.0				4.90	9.3	26.5
	481	4.60	8.8	33.0	5.1	10.6	32.0						

*All monkeys inoculated intracerebrally with virus.
†Millions per cubic millimeter.
‡Grams per 100 milliliters.
§Per cent whole blood.

TABLE VII. NORMAL AND TREATED RHESUS MONKEYS
TOTAL WHITE BLOOD CELLS (THOUSANDS PER CUBIC MILLIMETER)

		4/27/50	5/3/50	5/11/50*	5/16/50
Virus Control	65	13.9	15.6		9.4
	66	33.2	18.8		10.4
	67	22.8	17.2		17.4
	68	19.6	25.2		13.2
	69	12.2	16.0		14.6
	82	14.2	18.0		13.2
	64	10.6	10.8		
	69a	13.2			
ACTH daily 5/8–5/11	70	24.9	29.2		12.2
	72		12.8	27.2	21.8
	73	15.2	13.6	16.8	19.0
	77	10.8	14.6		12.4
	78	20.0	14.0	26.8	15.0
	79	13.9	9.6	19.6	16.0
ACTH daily 5/8–5/16	71	14.9	10.6	19.8	16.0
	74	17.9	13.0		12.4
	75	21.0	20.4		19.8
	76	12.2	10.0	27.8	15.6
	80	15.6	13.2		15.8
	81	14.4	10.8		

*All monkeys inoculated intracerebrally with virus.

TABLE VIII. NORMAL AND TREATED RHESUS MONKEYS
DIFFERENTIAL WHITE BLOOD CELL COUNTS*

	K NO.	4/27/50					5/3/50					5/11/50†					5/16/50				
		PMN	LYM.	MONO.	EOS.	BASO.	PMN	LYM.	MONO.	EOS.	BASO.	PMN	LYM.	MONO.	EOS.	BASO.	PMN	LYM.	MONO.	EOS.	BASO.
Virus Control	465	50	46	3	1	0	74	26	0	0	0						59	38	3	0	0
	466	64	26	4	6	0	64	32	1	3	0						40	40	10	9	1
	467	59	34	4	3	0	63	35	2	0	0						38	51	6	5	0
	468	49	47	3	1	0	51	47	1	1	0						60	31	4	4	1
	469	56	36	5	3	0	52	36	10	1	1						46	40	12	2	0
	482	19	67	2	10	2	33	62	3	2	0						26	65	3	5	0
	464	42	51	6	1	0	72	28	0	0	0										
	469a	63	33	2	2	0															
ACTH daily 5/8–5/11	470	54	44	1	0	1	61	36	3	0	0						57	42	1	0	0
	472						50	47	2	0	1	93	6	1	0	0	69	27	3	0	1
	473	62	32	3	1	2	60	34	2	3	1	81	17	2	0	0	69	29	2	0	0
	477	56	37	5	2	0	64	32	4	0	0						57	34	8	1	0
	478	74	15	2	9	0	64	29	0	7	0	93	4	3	0	0	73	17	5	5	0
	479	41	51	7	0	1	43	56	1	0	0	94	3	2	1	0	87	13	0	0	0
ACTH daily 5/8–5/16	471	42	53	3	1	1	44	49	4	2	1	84	12	4	0	0	88	10	2	0	0
	474	38	57	3	2	0	45	50	2	3	0						72	22	4	2	0
	475	34	60	4	0	2	57	35	4	4	1						78	18	4	0	0
	476	54	40	3	1	2	30	67	1	3	0	89	9	2	0	0	93	5	2	0	0
	480	27	61	6	5	1	45	50	1	2	2						85	12	3	0	0
	481	31	64	4	0	1	60	37	2	0	1										

*Expressed in per cents of 200 WBC counted from stained blood smears.
†All monkeys inoculated intracerebrally with virus.

There were no significant changes in the glucose tolerance curves (Table XI) of eighteen monkeys during the first experiment, nor in their blood pictures (Tables V to VIII) except for those discussed above.

TABLE IX. NORMAL AND TREATED RHESUS MONKEYS
TOTAL CIRCULATING EOSINOPHILS PER CUBIC MILLIMETER

EXPERIMENT 1						EXPERIMENT 2						
MONKEY		DATE				MONKEY		DATE				
GROUP	NO.	4/27	5/3	5/11*	5/16	GROUP	NO.	8/18	8/21	9/7	9/14*	9/23
Virus Control	465	22	33	6		Virus Control	548	289	255	155	266	111
	466	300	744		28		549	555	344	511	444	278
	467	33	178		17		550	766	577	289	411	255
	468	44	225		34		551	555	588	488	489	
	469	56	133		78		552	377	366	366	356	289
	482	655	844		309		553	400	411	400	411	522
	464	22	33									
	469a	22										
ACTH daily 5/8–5/11	470	22	11		0	ACTH daily 9/11–9/14	541	244	544	389	67	78
	471		89	0	6		542			810	133	100
	473	67	122	2	0		543	189	333	744	67	
	477	56	33		0		544	355	333	422	67	89
	478	189	755	2	217		545	344	244	344	44	189
	479	22	122	4	44		546	266	189	644	11	
ACTH daily 5/8–5/16	471	22	44	2	11	ACTH daily 9/11–9/23	536	111		389	166	67
	474	44	111		6		537	255	244	466	33	
	475	56	167		6		538	654	433	500	56	44
	476	111	56	2	34		539	444	455	255	11	
	480	189	466		0		540			810	22	
	481	78	78				547	244	155	278	0	0

*Virus or normal cord suspension inoculated intracerebrally.

TABLE X. CYNOMOLGUS MONKEYS
TOTAL CIRCULATING EOSINOPHILS PER CUBIC MILLIMETER

EXPERIMENT 3									
MONKEY		DATE							
GROUP	NO.	12/8*	12/9	12/11	12/13	12/15	12/18	12/20	1/3/51
Normal Uninoc. Control	689	866	477	322	477	477	722	655	344
	590	555	588	333	433	366	810	566	477
	691	344	433	695					
Normal Cord S.Q.	683	432	278	289	555	499	444	622	521
	684	710	588	268	544	433	278	433	511
	685	455							
Normal Cord I.C.	686	422	255	200	333	211	444	295	455
	687	866	687	444	799	499	455	333	700
	688	632	411	611	533	366	479	333	810
Virus Oral	673	1021	534	422	555	444	477	555	810
	674	766	687	1210	877	655	544	355	433
	675	555	832	378	366	255	377	466	600
	676	632	644	466	455	477	411	366	721
Virus S.Q.	677	70	355	455	344	178	477		
	678	688	544	233	222	233			
	679	710	466	289	299	178			
Virus I.C.	680	822	500	211	278	200			
	681	422	288	222	222	289	278	322	455
	682	599	422	433	433				

*Virus or normal cord suspension inoculated or fed.

TABLE XI. NORMAL AND TREATED RHESUS MONKEYS
GLUCOSE TOLERANCE TESTS*

	K NO.	4-21				5-3				5-11†				5-16			
		FAST	1 HR.	2 HR.	3 HR.	FAST	1 HR.	2 HR.	3 HR.	FAST	1 HR.	2 HR.	3 HR.	FAST	1 HR.	2 ER.	3 HR.
Virus Control	464	75	90	93	95	102	162	214	191								
	465	100	122	107	82	102	94	93	105					105	107	156	92
	466	109	164	105	94	97	96	100	95					122	128	88	67
	467	83	102	85	73	91	91	85	102					96	93	99	87
	468	76	180	141	55	104	130	113	123					85	89	89	96
	469	86	284	193	87	117	175	135	67					96	138	73	84
	482	103	158	106	68	87	73	63	89					99	105	78	93
ACTH daily 5/8–5/11	470	83	146	89	67	103	110	123	103					73	106	95	95
	472					98	111	95	73	97	90	83	70	117	79	102	81
	473	117	148	123	71	92	99	84	79	100	136	93	75	79	83	169	74
	477	85	78	81	67	86	134	97	66					99	119	101	95
	478	86	201	107	60	78	143	80	86	101	217	133	92	90	158	104	68
	479	90	89	57	85	103	111	80	88	89	144	139	55	80	135	46	83
ACTH daily 5/8–5/16	471	85	104	59	70	91	75	73	74	97	90	83	70	94	181	148	64
	474	84	81	75	71	76	96	52	75					79	83	64	71
	475	97	212	138	86	80	103	76	102					92	102	98	76
	476	75	80	110	83	86	118	87	101	92	91	103	114	95	148	77	69
	480	89	131	92	101	91	104	106	136					84	124	73	85
	481	63	139	125	116	75	124	114	61								

†All monkeys inoculated intracerebrally with virus.
*Three grams glucose per kilogram as a 20 per cent solution given orally.

From Table IV it can be seen that in the preliminary experiment the 17-ketosteroids of the urine showed a three- to fourfold rise during the administration of ACTH. In this monkey the total eosinophil count dropped from an average of 146 per cubic millimeter to between 0 and 5 on the third to seventh days, and during the administration of ACTH the neutrophil and lymphocyte differential counts changed from averages of 55 per cent and 39 per cent to 86 per cent and 12 per cent, respectively. There were no significant changes in the levels of fasting blood sugar or in the glucose tolerance curves during this period.

DISCUSSION

The average eosinophil counts of normal monkeys showed a variation of approximately 50 per cent from the grand average which is similar to the variation observed in normal human subjects.[9-11] Although a depression of the eosinophil count below the normal range was observed in monkeys which developed poliomyelitis, a far greater and more prolonged depression was effected by the administration of ACTH. The latter observation, as well as the three- to fourfold increase in the excretion of 17-ketosteroids in the urine of one monkey during the administration of ACTH, demonstrate that the dosage used was sufficient to elicit a definite physiologic response.[8, 9, 11] The rise in eosinophils when ACTH was discontinued indicates that the adrenal was not exhausted by the administration of 100 mg. of ACTH daily for three days. Thus the attempt to have one group of monkeys serve to test the effect of relative adrenal insufficiency was not as successful as had been hoped. The eosinophil response also demonstrated that at no time following the inoculation of an infective suspension of poliomyelitis virus was there as marked a stimulation of the adrenal cortex as that which resulted from the daily administration of 100 mg. of ACTH. Since maximal stimulation of the adrenal would be expected prior to its exhaustion, the results indicate that adrenal failure is not a factor in the occurrence of paralytic poliomyelitis in monkeys. This conclusion is supported by similar results reported in a recent clinical study of poliomyelitis[12] in man. It was found that patients tested an average of 2.6 days after onset of illness had a depressed eosinophil count which was further depressed by the administration of ACTH.[12] The results of studies on monkeys and human subjects with poliomyelitis indicate, therefore, that not only is exhaustion of the adrenal cortex unlikely, but that the stimulation which does occur is only of moderate degree.

Results with monkeys further suggest the possibility that stimulation by exogenous ACTH results in a decreased resistance to the infection, insofar as there was a slightly shorter average pre-paralytic incubation period in 3 of 4 groups treated with ACTH. Thus, the increased susceptibility to, or severity of, paralytic poliomyelitis in the following conditions might be related to the adrenocortical stimulation which accompanies each one: pregnancy in women[13, 15] and mice,[16, 17] excessive fatigue during the incubation period in monkeys[18] and man[19, 20] and probably following the surgical procedures[10] of tonsillectomy in man[21, 22] and monkeys,[23] and possibly following other operations which appear

to result in a similar incidence of severe poliomyelitis.[24] These observations indicate that hormonal influences may play a part in the occurrence of clinical poliomyelitis as has been suggested before.[25, 25a]

Many observations are available which indicate that the defense mechanisms of the body are not in a normal state after the administration of ACTH or cortisone. Examples include reports of the inhibition of allergic inflammatory responses,[26, 27] increased susceptibility to bacterial toxins,[28] increased severity of experimental tuberculosis,[29, 30] syphilis,[31] and streptococcosis,[32] and of pneumococcal pneumonia in man.[33]

Subsequent to preparation of this manuscript an article by Schwartzman[34] appeared in which it was reported that there was a marked increase in the susceptibility to poliomyelitic infection when the relatively refractory hamster was treated with cortisone, or cortisone combined with ACTH, but not when treated with ACTH alone. Acceleration of the infection was also noted in an experiment with nine mice. Both drugs were given in doses of five milligrams. The mice received one injection of ACTH followed by two injections of cortisone at intervals of four and nineteen hours, the virus being inoculated two hours after the injection of ACTH. The divergence between the results reported in the experiment in mice and the present results in mice (Table I) cannot be evaluated because of the differences in the character of the experiments. It seems of interest, however, to note that in the first experiment of the present report the administration of cortisone, or of ACTH, also resulted in a shortening of the incubation period, although not to a marked extent, but that subsequent experiments were negative. The effect of cortisone in hamsters agrees with other observations that its administration results in an increased severity of infection,[26-34] but the negative results with ACTH remain unexplained.*

SUMMARY

In each of two experiments ACTH was given in doses of 25 mg. every six hours to twelve rhesus monkeys for three days before intracerebral inoculation of the Minnesota strain of poliomyelitis virus, and continued in 6 of the animals until the onset of paralysis. Six controls in each experiment received virus, but no hormone. A physiologic response was obtained with the hormone as shown by the precipitous drop and subsequent low level in the number of circulating eosinophils, and by the increased excretion of 17-ketosteroids in the urine but not by any changes in the fasting blood sugar values, or glucose tolerance curves. All twenty-four monkeys treated with ACTH, and ten of twelve controls, developed poliomyelitis. In four of twelve monkeys receiving continuous treatment paralysis occurred on the fifth day, two to three days earlier than the first paralysis in the twelve controls. Paralysis occurred 0.6 or 2.6 days earlier in the groups on continuous treatment than in the controls, and 0.0 or 1.0 day

*At the time of submission of this manuscript an article by Milzer[35] appeared in which it was reported that 1.0 mg. per day of ACTH administered to mice in two equally divided doses starting one day before inoculation of virus had no protective effect upon infection with the Lansing strain of poliomyelitis virus or the B. A. I. strain of Western equine encephalomyelitis virus. Also, no therapeutic effect was obtained when the drug was started after the onset of either disease.

earlier in the groups treated only before inoculation with the virus. This suggests that the administration of ACTH not only fails to induce increased resistance to the virus, but appears to render the host slightly more susceptible.

In these experiments a significant drop in circulating eosinophils was noted to follow the inoculation of the virus suspensions in rhesus monkeys which did not receive ACTH. In a third control experiment with cynomolgus monkeys, the inoculation of infected spinal cord by either the intracerebral or subcutaneous routes also resulted in a similar decrease, while the inoculation of a suspension of normal cord did not have this effect. However, the decrease after inoculation of infected material was of lesser magnitude and shorter duration than that resulting from treatment with ACTH.

CONCLUSIONS

The administration of ACTH to rhesus monkeys inoculated with poliomyelitis virus resulted, at the most, in a slight acceleration of the onset of paralysis which was most suggestive in the groups which received ACTH both before and after the inoculation of virus. There was a significant though moderate decrease in the total eosinophil counts of rhesus and cynomolgus monkeys after inoculation of poliomyelitis virus by the intracerebral or subcutaneous routes, but not after the inoculation of a suspension of normal spinal cord. Observations are also reported on the blood picture and glucose tolerance curves of normal and treated monkeys.

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THE INFLUENCE OF ACTH ON THE RESISTANCE OF MONKEYS TO THE YALE SK STRAIN OF POLIOMYELITIS VIRUS

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THE problem of low incidence of paralysis in spite of high rates of infection with the virus of poliomyelitis has been the subject of considerable study and extensive speculation. It is obvious from many past investigations that metabolic and other physiologic factors such as stress may influence the development of overt disease.

In the summer of 1949 when ACTH became available for investigative purposes, it seemed possible that its use might provide some information on the relationship between such host factors and the course of poliomyelitis. At this time Coriell and his co-workers[1] found no apparent improvement of treated cases of poliomyelitis over the controls when ACTH was added in the therapy of children in whom signs had already developed. The present report is concerned with a study of the influence of the physiologic state of the host, as altered by ACTH, at the same time of infection, rather than after onset of signs in experimentally induced disease. The length of incubation period and incidence of overt disease in the treated and control groups were the criteria used in determination of the effect of the changed physiologic state.

In order to make this study as comparable as possible to the pattern of illness in man, it was decided to use monkeys and to employ a route of inoculation which would cause overt illness in a relatively small number of animals. Rhesus monkeys were injected, therefore, with virus by the intratonsillar route.

MATERIALS AND METHODS

Monkeys.—Fifteen Rhesus monkeys received from the Okatie Farm were maintained in this laboratory for one week before treatment during which time all appeared to be well. One (monkey No. 13 in the experiment) was used for a preliminary test to determine the proper dose of ACTH to be used.

Two others were used for virus passage, but only one of them developed signs of infection. This animal was sacrificed and his brain and cord used as a source of virus. The remaining twelve monkeys, together with No. 13 used for dosage test were divided into two groups, six receiving ACTH and six saline. Monkey No. 13 was the 17th animal in the ACTH group. All were injected with the virus. They were housed in pairs, one ACTH-treated and one saline-treated monkey being kept in the same cage.

Virus.—The stock culture of Yale SK strain of poliomyelitis virus was received from Dr. Joseph Melnic. A 15 per cent suspension of the brain and cord of the monkey infected in this laboratory with this stock culture served as the inoculum in the experiment here reported. Both groups of monkeys received a 0.2 ml. dose into each tonsil twenty-four hours after the first dose of ACTH had been given to the treated group.

ACTH.—The product used was supplied by Armour and Company. It was diluted with double distilled water to yield 10 mg. per milliliter on the basis of the potency indicated on the label. A preliminary test to determine an effective dose in monkeys was performed

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in one monkey as stated previously. The results in this one animal suggested that a good response might be expected with a dose of 10 mg. every six hours (see Fig. 1). This dosage was given at the start of the experiment, and it was hoped that lower doses would then be effective. However, it was found that 10 mg. every six hours (± one hour) by intramuscular injection were necessary, and this regimen was continued until the onset of illness. (For details of dosage see Table I.) In the case of monkey No. 1 which showed

TABLE I. DAILY TOTAL MG. OF ACTH

Days in relation to inoculation	−1	0	1	2	3	4	5	6	7	8	9	10	11	12
Mg. ACTH	40	30	40	27	35	20	40	40	40	40	40	40	40	40

no signs of disease ACTH was administered for seventeen days. From the third post-inoculation day the monkeys which did not receive ACTH were given an equivalent volume of 0.85 per cent NaCl. Otherwise, the handling of the two groups was the same.

Eosinophil Counts.—These counts were made every twenty-four hours according to the method described by Randolph.[2]

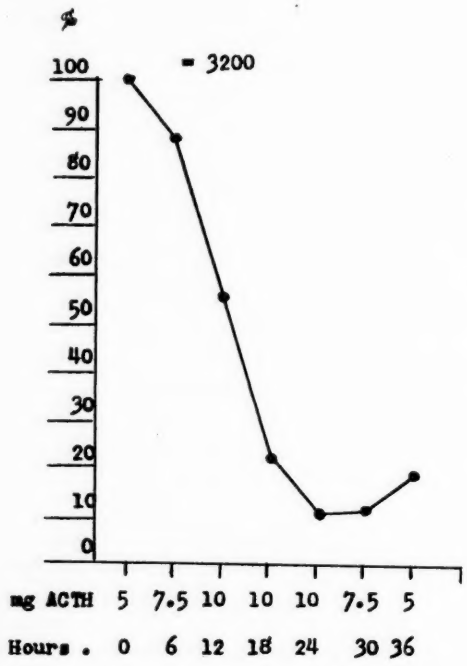

Fig. 1.—Eosinophil counts on blood from the thenar eminence taken immediately before ACTH injection indicated.

OBSERVATIONS

Four times daily each monkey was observed clinically as he moved about in the cage immediately preceding the injection with ACTH.

Daily eosinophil counts on monkeys receiving ACTH revealed that the degree of change was irregular, ranging from insignificant depression to complete disappearance of eosinophils. With a 50 per cent decrease in eosinophils as an arbitrary threshold, and basing the frequency of significant eosinophil depression on the number of counts 50 per cent or more below the count taken immediately before the first ACTH dose, the frequency of significant depressions was found to vary from 66 to 100 per cent of the total daily counts.

As might have been expected, there was an increase in total eosinophils when the ACTH dose was decreased and when the midnight dose was omitted, leaving a twelve-hour interval between the last dose on the fourth day and the first dose on the fifth day after inoculation. With a return to larger dosage the eosinophils were again reduced. In all the paralyzed monkeys in the control as well as the treated group the counts were very low (2 per cent to 23 per cent of normal) on the day of onset of paralysis.

Table II summarizes all results. It can be seen that six of seven animals in the ACTH group became paralyzed between the sixth and ninth days. The average incubation period in this group was 7.8 days. In the control group two of six animals were paralyzed. Their incubation period was nine days. Two other monkeys appeared to be ill on the eleventh and thirteenth days, respectively, although only one of these (sick on the thirteenth day) showed characteristic histopathologic evidence of poliomyelitis. Both appeared to have recovered by the sixteenth day after inoculation, when the experiment was terminated. If all these animals are considered as suffering from poliomyelitis, the range is between nine and thirteen days, or an average of 10.5 days. However, it is of importance to emphasize the different incidence of paralysis in the two groups. It is of especial interest to note that among the three animals that developed paralysis or observable histopathologic changes in the control group there was a depression of eosinophils in 60 to 75 per cent of the counts as compared with only 15 to 23 per cent among those without paralysis or observable histopathologic changes.

TABLE II

MONKEY NO.	EO Δ*	DAY OF ONSET OF ILLNESS†	RANGE OF INCUBATION PERIOD	PATHOLOGIC FINDINGS
		A—ACTH-Treated Monkeys		
1	10/15	‡		—
2	5/7	7		n.d.
3	8/9	9		+
4	6/9	8	6-9	n.d.
5	6/9	9		n.d.
6	5/6	6		n.d.
13	7/7	8		n.d.
		B—Saline-Injected Monkeys		
7	6/8	9		+
8	2/13	11-S§	9-13	—
9	6/8	9		+
10	8/14	13-S§		+
11	3/13	‡		—
12	3/13	‡		—

*Denominator = total number of daily eosinophil counts; numerator = number of those counts in which there was a 50 per cent or greater drop in eosinophils.

†In days after virus inoculation; paralysis except in case where an "S" (meaning debility only) is placed after the number.

‡ = No signs of illness during the experiment.

§ = Showed no signs of poliomyelitis up to the sixteenth day after inoculation.

DISCUSSION

Since the completion of this study Ainslie, Francis, and Brown[3] have made similar observations on monkeys, and their findings suggest that the action of ACTH increases the susceptibility of this host to the virus of poliomyelitis. Schwartzman,[4] on the other hand, reported that cortisone but not ACTH increased the susceptibility of hamsters to the poliomyelitis virus.

Although the number of animals is too small to permit definite conclusions, the data here presented suggest that ACTH may increase the susceptibility of monkeys to paralytic poliomyelitis. The observations on the changes in eosinophils in the natural disease[1] associated with these findings opens another

experimental approach to the problem of the host-parasite relationship in this disease, and may lead to a better understanding of the varied reaction of the host to its frequent encounter with poliomyelitis virus.

SUMMARY

ACTH induces a decrease in eosinophils in the monkey. The change in counts is often irregular but a significant depression can be maintained with adequate dosage.

In most instances, the counts in the ACTH-treated animals were at their lowest on the day of onset of paralysis or immediately preceding it.

Those animals in the control series which became paralyzed or showed histopathologic evidence of poliomyelitis also had a significant depression of eosinophils in the majority of their daily counts.

Whereas in the ACTH group six of seven monkeys became paralyzed, only two of six developed paralysis in the control group.

The results, although not conclusive, lend some support to the belief that the adrenal may play an important role in respect to paralysis in poliomyelitis infection.

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THE EFFECT OF CORTISONE IN STREPTOCOCCAL LYMPHADENITIS AND PNEUMONIA

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RECENT studies relating to the action of the adrenal steroid 17-hydroxy-11-dehydrocorticosterone (cortisone) and of pituitary adrenocorticotrophic hormone (ACTH) have revealed that they exert powerful and, in many respects, similar effects upon a wide variety of diseases. The experiments described in this report deal with the effects of cortisone in experimental infections due to group A streptococci, with particular reference to alterations in the response of the host to bacterial invasion.

Finland and co-workers,[1] in a preliminary study, described remarkable alterations in the course of type 8 pneumococcal pneumonia during treatment with ACTH. Within eighteen hours after therapy was instituted, the patient's temperature fell to normal and he felt well; nonetheless, a blood culture drawn thirty-six hours after the start of treatment was positive, and type 8 pneumococci persisted in the sputum for many days. In a recent more detailed report these same authors[2] described a second patient with type 2 pneumococcal pneumonia treated with cortisone, who produced rusty sputum until the eighth hospital day; type 2 pneumococci were cultured from the sputum for ten days. This patient, like the first one, showed dramatic clinical improvement despite the persistence of organisms in his sputum. In a third patient with type I pneumococcal pneumonia the organisms persisted in the sputum despite a slower but distinct improvement in symptoms. Subsequently the patient again became febrile and was found to have developed empyema.

In human pulmonary tuberculosis, ACTH has had, if anything, a deleterious effect,[3] and in rats,[4] guinea pigs,[5] and mice[6] cortisone has likewise been identified with enhancement of tuberculous infection.

Intradermal group A streptococcal infections in rabbits, which have not led to death in control animals, have been rendered uniformly fatal in cortisone-treated rabbits.[7]

The influence of cortisone on inflammatory responses has attracted the attention of a number of investigators. It has been observed that the steroid delays the development of granulation tissue in rabbits,[8] in rats,[9] and in mice.[10] Similarly, vascularization of the damaged cornea of the rabbit has been inhibited.[11] Although the acute inflammatory response to turpentine in mice was not affected by cortisone,[10] the response to intraperitoneal injection of mineral oil in the rat was definitely modified.[4] Inhibition of the Shwartzman phenomenon in rabbits has been described.[12] In the same animal the Arthus reaction may be prevented by cortisone,[13] and the vascular lesions of serum sickness may be significantly altered.[14, 15]

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METHODS

Cervical adenitis was produced in pure strain white mice with group A streptococci (strains C203 and GL8), employing a modification[16] of the intranasal method of Sonkin.[17] Pneumococcal pneumonia was induced in albino rats by the technique previously described;[18] type I organisms (A5 strain) were used. Experimental streptococcal pneumonia (strain GL8) was produced by the same method.[19] In all experiments four-hour cultures of the infecting organisms were used.

In early experiments cortisone* (Cortone acetate, Merck) was given at the time of inoculation, and thereafter as indicated in the protocols. Subsequently, because of the observation of Ebert,[14] cortisone was begun five days prior to inoculation. The pneumonic lungs of animals that died or were sacrificed were prepared for histologic study by standard techniques already outlined.[18] Hearts' blood was used for culture in the mouse experiments; in rats, blood for culture was secured from the axillary vein by aseptic technique except in the bacteremia experiment where it was obtained from the tail vein. Cultures were made on the surfaces of blood agar plates. The data from the various mortality studies were subjected to statistical analysis; in each case the results were found to be statistically significant.†

TABLE I

Experiment	*Difference of Means* / *Standard Error of Difference*
Streptococcal lymphadenitis—cortisone begun at time of inoculation†	2.68
Streptococcal lymphadenitis—cortisone begun 5 days prior to inoculation	3.18
Pneumococcal pneumonia—cortisone begun 5 days prior to inoculation†	2.60
Streptococcal pneumonia—cortisone begun 5 days prior to inoculation	3.40

*Values of 2.5 or above are significant at the 1 per cent level and those of 3.0 or above at the 0.2 per cent level.

†In each of these experiments two control animals survived; for purposes of the analyses they were disregarded. As a result, the values given for these two experiments, while significant, are obviously lower than they would have been had arbitrary survival values been assigned to the animals which recovered.

RESULTS

I. *Cortisone Treatment of Streptococcal Cervical Adenitis in Mice.—*

(a) *The Effect on Mortality Rate of Cortisone Treatment Begun at the Time of Inoculation:*

Twenty mice were inoculated intranasally with the C203 strain of group A streptococci. Immediately after inoculation and once daily for the remainder of the experiment ten mice received 2.5 mg. of cortisone intraperitoneally; the other ten mice served as controls. The time of death of each animal was recorded, and blood cultures were obtained at autopsy.

In Fig. 1 the mortality curves for both groups of animals are plotted. The cortisone-treated animals succumbed to the infection at a more rapid rate than did the controls; in addition, two control animals survived, whereas all of the cortisone group died. All of the blood cultures in both groups were positive.

*The cortisone used in the preliminary aspects of this study was allocated by the Committee on Cortisone of the National Academy of Sciences.

†Dr. Robert E. Shank of the Department of Preventive Medicine reviewed the experimental data and gave valuable advice in regard to the analyses, the resluts of which are summarized in Table I.

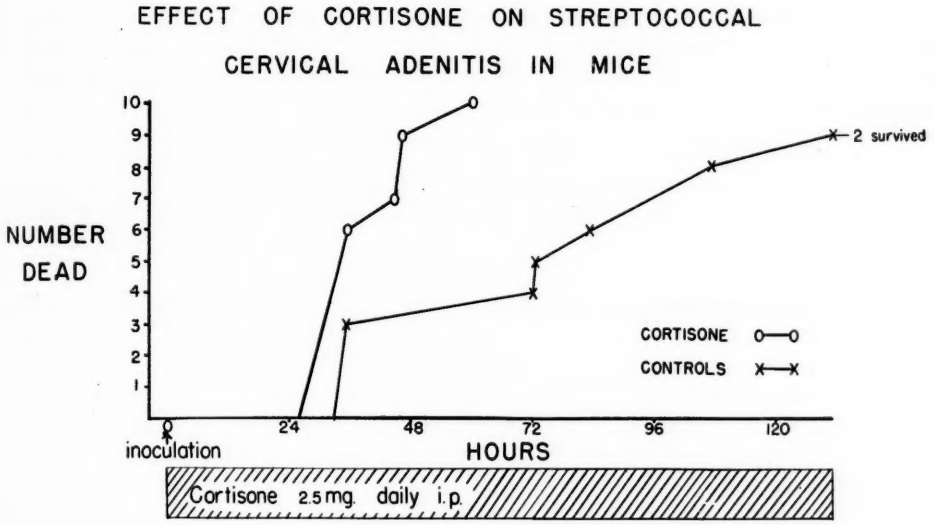

Fig. 1.—Mortality rates of streptococcal cervical adenitis in mice treated with cortisone from the time of infection and in untreated controls.

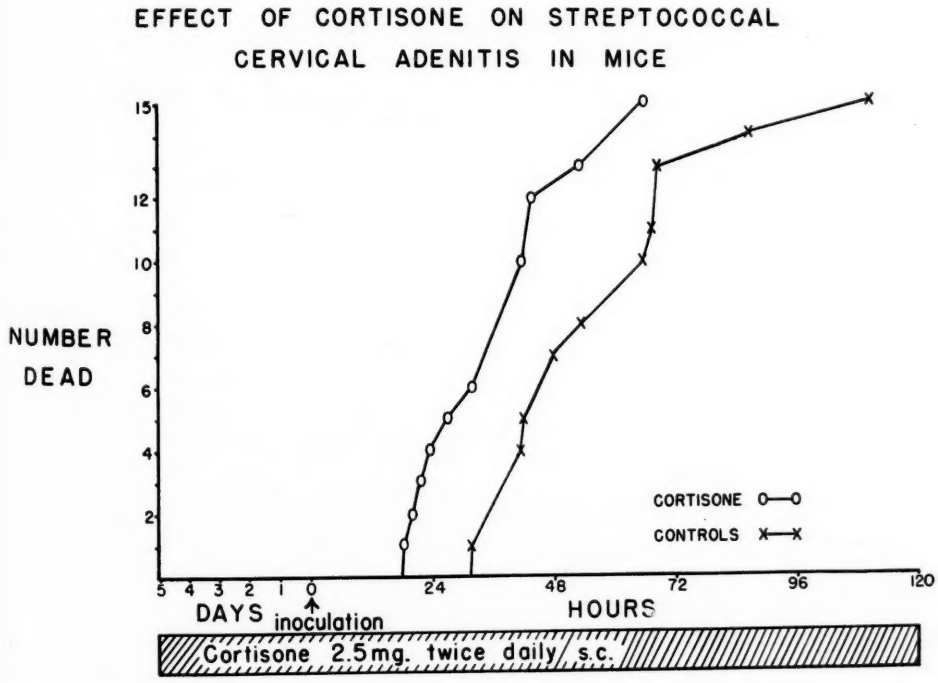

Fig. 2.—Mortality rates of streptococcal cervical adenitis in mice pretreated with cortisone and in untreated controls.

(b) *The Effect of Pretreatment With Cortisone:*

Thirty mice were infected by the intranasal route employing the GL-8 strain. Fifteen animals were given 2.5 mg. of cortisone subcutaneously daily beginning five days prior to inoculation; the same dose was continued throughout the course of the experiment. The fifteen untreated mice served as controls.

The results of this experiment are shown in Fig. 2. The mortality curve of the cortisone-treated animals was less favorable than that of the untreated controls, although ultimately all the mice in both groups died. All had positive blood cultures.

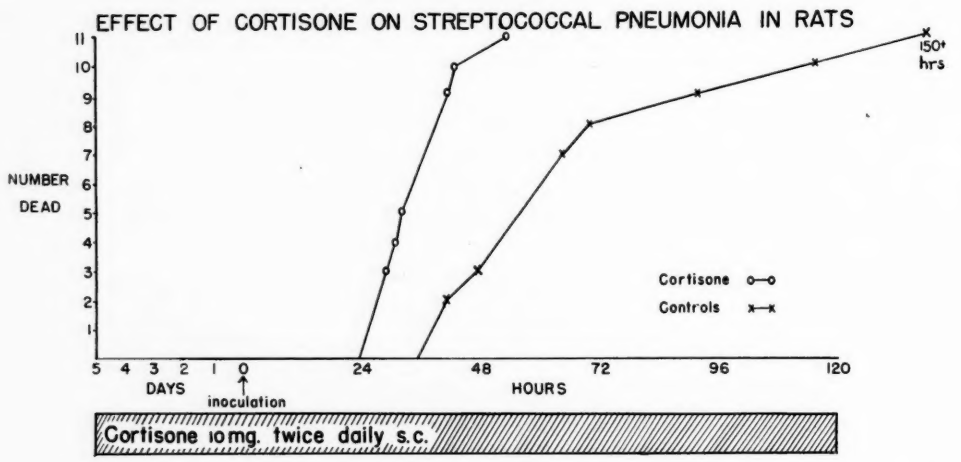

Fig. 3.—Mortality rates of streptococcal pneumonia in rats pretreated with cortisone and in untreated controls.

II. *Cortisone Treatment of Streptococcal Pneumonia in Rats.*—

(a) *The Effect of Cortisone on the Mortality Rate:*

Twenty-two albino rats were infected by the intrabronchial route with approximately 2,600 streptococci; half of the animals were given 5 mg. of cortisone twice daily intramuscularly, beginning five days prior to inoculation and continuing throughout the course of the experiment. The other eleven were untreated and served as controls.

The time of death of each rat was noted. An autopsy was performed, and the extent and character of the pulmonary lesion recorded; in instances when the autopsy was done immediately after the animal's death, blood cultures were made and the lungs were removed and prepared for histologic study.

The results of this experiment are shown in Fig. 3. The rats treated with cortisone exhibited a less favorable survival rate than did the untreated controls;* indeed, only 50 per cent of the controls had died by the time all of the cortisone-treated animals had succumbed. Further, many of the gross lesions in the cortisone group appeared excessively hemorrhagic, and frequently the pleural effusions were bloody, whereas in the control animals they were not. In both groups all post-mortem blood cultures obtained were positive.

*It is of interest that the mortality curve of pneumococcal pneumonia in rats (type I, strain A5) was also adversely affected by cortisone treatment—see Fig. 4.

The limited histologic observations in this mortality experiment suggested that the following differentiating features characterized the pneumonic process in the cortisone group: (1) the edema reaction was intensified; (2) the number of bacteria was greatly increased; actual colony formation was frequently seen; (3) the appearance of the polymorphonuclear leucocytic exudate was delayed although, when leucocytes were present, phagocytosis was unimpaired.

In an attempt to define further the effect of cortisone on the character and course of group A streptococcal pneumonia additional pathogenesis experiments were performed.

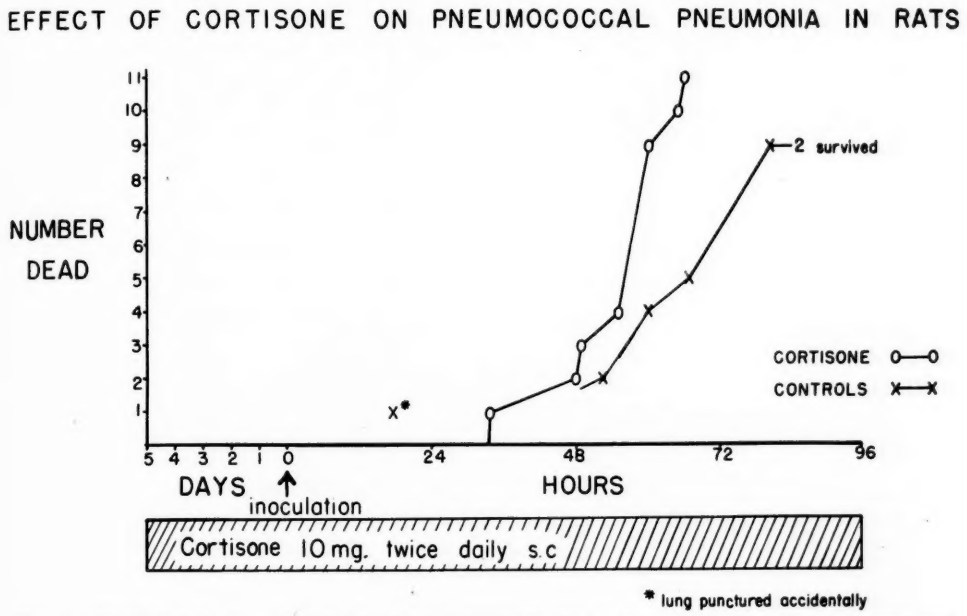

Fig. 4.—Mortality rates of pneumococcal pneumonia in rats pretreated with cortisone and in untreated controls.

(b) *The Pathogenesis of Streptococcal Pneumonia in Cortisone-Treated Rats:* A number of experiments was performed in which animals were sacrificed at various time intervals after inoculation and their lungs prepared for histologic study.

Fifteen rats were pretreated for five days with 5 mg. of cortisone twice daily; an equal number of untreated rats served as controls. All of the animals were inoculated with 9,200 organisms, and three from each group were sacrificed at each of the following intervals after infection: six, twelve, eighteen, twenty-four, and thirty-six hours.

Differences in the histologic findings in the two groups of animals were most striking at eighteen and twenty-four hours after inoculation. The edema reaction in the cortisone-treated rats was much more extensive and the polymorphonuclear response less prominent (Fig. 5, *1*) than in the control animals (Fig. 5, *2*). Within the edema fluid in the cortisone-treated group there were myriads of bacteria (Fig. 5, *3*), whereas in the controls the streptococci were comparatively scarce (Fig. 5, *4*). Along the pleural edge of the involved lung

in the treated animals, tremendous bacterial growth with definite colony formation was observed (Fig. 5, *5*). In comparable areas of the pneumonic lesion in untreated animals, the infection appeared to be under control most of the bacteria had been phagocyted, and the exudate was predominantly mononuclear in character (Fig. 5, *6*). In some areas of the lesion in the cortisone animals, sheets of bacteria filled the alveoli (Fig. 5, 8), and necrosis of tissue was apparent (Fig. 5, *7*).

Because of the extreme virulence of the organism used in this study, relatively fulminating lesions were produced in the control animals; nonetheless, the pneumonic process appeared to be significantly more malignant in the cortisone-treated animals.

Additional experiments were performed in which the inocula were decreased to one-tenth those previously employed. Animals in both the cortisone-treated and the control groups were sacrificed approximately eighteen and twenty-four hours after inoculation.

The findings were essentially the same as those recorded above. Although individual variations in the histologic appearance of the pneumonic lesions were again noted within each group, the over-all results were consistent with the conclusion that the cortisone-treated rats suffered a more fulminating infection than did the untreated controls.

(c) *The Effect of Cortisone upon Invasion of the Blood Stream:*

Twelve rats were treated with 5 mg. of cortisone subcutaneously twice daily for five days and then inoculated intrabronchially with 450 streptococci; ten untreated rats, infected at the same time with an identical inoculum, served as controls. At given intervals, tail vein blood was obtained for culture from all surviving animals in each group.

TABLE II. EFFECT OF CORTISONE ON THE INCIDENCE OF BACTEREMIA IN RATS WITH STREPTOCOCCAL PNEUMONIA

HOURS AFTER INOCULATION	CORTISONE TREATED ANIMALS			CONTROL ANIMALS		
	NUMBER SURVIVING	NUMBER WITH BACTEREMIA	PER CENT WITH BACTEREMIA	NUMBER SURVIVING	NUMBER WITH BACTEREMIA	PER CENT WITH BACTEREMIA
18	12	2	16.6	10	0	0
26	12	1	8.3	10	0	0
30	12	6	50.0	10	2	20
43	9	5	55.5	6	0	0
46	7	6	85.7	6	0	0
71	0	0	0	5	0	0
90	0	0	0	1	0	0

The results of this experiment are shown in Table II. It is seen that even with the very small inoculum employed, the cortisone animals developed bacteremia much earlier than did the controls; indeed, in the latter group, bacteremia was a terminal event. It can also be seen that at a time when all of the cortisone-treated animals had succumbed, half of the controls were still alive—further confirmation of the effect of cortisone on the mortality curve.

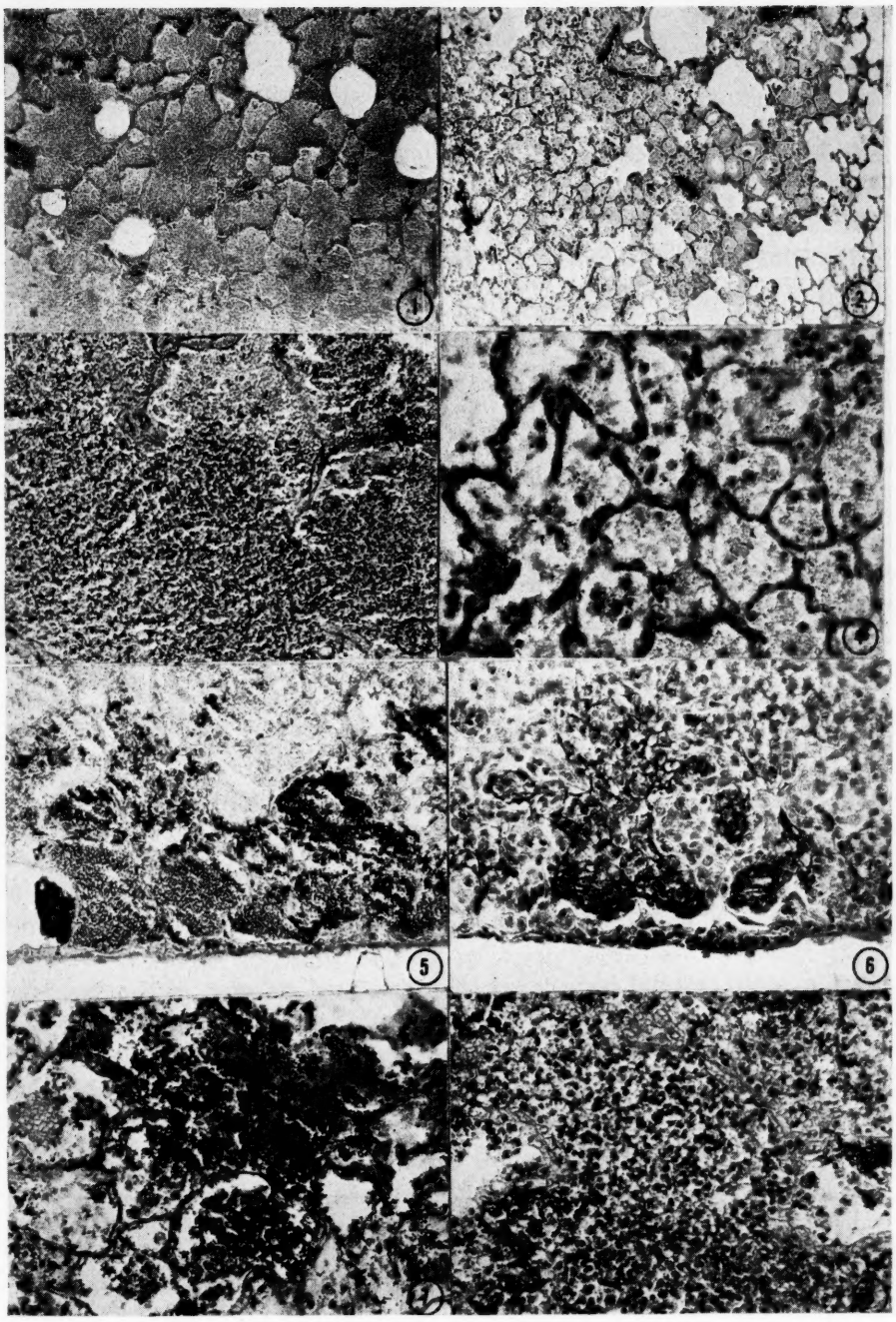

Fig. 5.—The photomicrographs were made by Mr. K. Cramer Lewis, Department of Illustration, Washington University School of Medicine. Comparable sections (*1* and *2*, *3* and *4*, and *5* and *6*) were obtained from animals infected at the same time with identical inocula. Sections stained by the Gram-Weigert technique.

1, Edema fluid in alveoli of cortisone-treated rat sacrificed eighteen hours after inoculation with group A streptococci. Compare alveolar distention in this section with that seen in *2*. (×92.)

2, Edema zone in the lung of a control rat 18 hours after induction of infection. (×92.)

3, A portion of the edema zone seen in *1* under higher magnification; myriads of bacteria are present. (×400.)

4, The edema zone of the control lesion in *2* under higher magnification. The number of streptococci is small when compared with the number seen in *3*, and polymorphonuclear leucocytes are apparent in most of the alveoli. (×400.)

5, Pleural edge of lesion in cortisone rat showing excessive bacterial growth. Actual colony formation is apparent. (×380.)

6, Comparable pleural edge of control lesion showing cellular exudate in which mononuclear cells are seen. Fibrin strands are prominent but few bacteria remain. (×380.)

7, Dense bacterial growth in the lung of a cortisone-treated rat sacrificed twenty-four hours after inoculation. Little or no cellular exudate is present. (×380.)

8, Tissue destruction in a cortisone lesion eighteen hours after induction of infection. (×400.)

It is also of interest to point out that in this experiment, the cortisone animals looked remarkably well for the first forty-two hours; in comparison, the control animals appeared toxic.

III. *Mechanism of Action of Cortisone.*—

Further studies were undertaken in an attempt to elucidate the mechanism by which cortisone increased the severity of the streptococcal pneumonia. Two general possibilities were considered: (1) that the steroid enhanced the growth of the bacteria, and (2) that the hormone in some way interfered with the cellular defenses of the host.

(a) *Possible Enhancement of Bacterial Growth:* An increased amount of edema fluid was observed repeatedly in the infected lungs of the cortisone-treated animals. Since the presence of alveolar edema fluid is known to favor bacterial growth,[20] this factor alone may have been partly responsible for the excessive numbers of bacteria in the lesions. The possibility that cortisone per se stimulated the growth of the streptococci was investigated as follows.

Cortisone acetate was added to serum broth in amounts calculated to yield final concentrations ranging from 0.02 mg. per milliliter to 5 μg per milliliter. Serum broth to which no cortisone was added served as a control medium. In a number of experiments, streptococci from eighteen-hour cultures were added to the various tubes and growth rates, as evidenced by turbidity, were observed over periods lasting up to eight hours.

The rate of growth of streptococci in the medium containing varying amounts of cortisone was the same as in the control medium. It was, therefore, concluded that cortisone per se had no effect on streptococcal growth in vitro.

(b) *Possible Interference with the Cellular Defenses of the Host:* When rats are exposed to total body irradiation and then infected with pneumococci, a relatively acellular pneumonia results;[21] the pneumonic exudate is characterized by much edema fluid, many bacteria and relatively few leucocytes. The effect of cortisone on circulating leucocytes was therefore investigated to determine whether depression of polymorphonuclear cells was responsible for the relative scarcity in the lesions.

The total numbers of circulating leucocytes were determined in eight rats. Four were then given 5 mg. of cortisone subcutaneously twice daily for five days, while the remaining four received control injections of sterile physiologic salt solution. At the end of the treatment period, the total leucocyte counts were again determined; differential counts were made, and the absolute number of polymorphonuclear leucocytes was calculated for each animal.

The results of this experiment are tabulated in Table III. It can be seen that although cortisone produced a leucopenic response, the leucopenia resulted from a fall in lymphocytes, the absolute number of polymorphonuclear leucocytes remaining essentially unchanged.

An alternative explanation for the relative scarcity of polymorphonuclear leucocytes in the pneumonic lesions of the cortisone-treated animals concerns a possible depressive action upon the diapedesis of leucocytes from the alveolar capillaries. Although no quantitative data are available regarding this possi-

bility, the number of polymorphonuclear leucocytes present in the pneumonic lesions appeared to be definitely decreased in the cortisone-treated animals. Since it was shown that there was no depression of these elements in the circulating blood, it appears reasonable to postulate that their diapedesis may in some way be inhibited by cortisone. Further studies relating to this point are now in progress.

TABLE III. EFFECT OF CORTISONE ON THE TOTAL LEUCOCYTE COUNTS AND ON THE ABSOLUTE POLYMORPHONUCLEAR LEUCOCYTE COUNTS IN RATS

TREATMENT	TOTAL LEUCOCYTES		PER CENT NEUTROPHILS	ABSOLUTE NUMBER OF NEUTROPHILS
	PRE-TREATMENT	POST-TREATMENT	POST-TREATMENT	POST-TREATMENT
Saline	1) 11,450	13,250	22	2,910
	2) 10,050	17,200	20	3,420
	3) 11,600	12,300	16	2,060
	4) 11,750	12,300	10	1,230
Cortisone	1) 8,650	2,300	70	1,610
	2) 6,900	4,300	89	3,820
	3) 5,000	3,500	76	2,660
	4) 12,900	4,500	83	3,730

DISCUSSION

Although the initial results with ACTH in bacterial pneumonia seemed spectacular, subsequent experiences have indicated the hazards which may be encountered where this agent is used. Recently, cortisone has been used in the treatment of bacterial pneumonia and has been found to mask the usual clinical picture without effecting control of the infection.[22] Cortisone has been identified with suppression of the clinical signs of an unrecognized, ultimately fatal pneumonic infection in a patient given the hormone for an unrelated disease.[23] The previously mentioned experimental studies of intradermal streptococcal infection in rabbits[7] also bear witness to the fact that this steroid may indeed be harmful.

The observations reported herewith lend weight to the thesis that cortisone may be dangerous in acute bacterial diseases, especially if given over a long period of time. It should be pointed out that in the present study relatively large doses of hormone were employed. Antopol[24] has already suggested on different grounds that excessive doses of cortisone may adversely affect the natural defenses of the host.

The increased rapidity with which cortisone-treated mice succumb to streptococcal cervical adenitis is clearly demonstrated in the present study. Similarly, the observations on streptococcal pneumonia indicate the detrimental effect of cortisone on survival rate. Although variations occurred from animal to animal, the striking histologic features in the latter experiments were: (a) the increased edema response, (b) the tremendous bacterial growth, and (c) the delayed appearance of the neutrophilic leucocytes in the lesion. Necrosis of pulmonary tissue was also common, probably because of the great number of bacteria in the lesion.[25] Importance may be attached to each of these findings,

but their relationship to each other cannot yet be defined. The normal response of the lung to acute bacterial infections involves the outpouring of edema fluid which in turn serves as a vehicle for spreading the infection.[26] The presence of edema fluid in the alveoli also provides a favorable medium for bacterial growth.[20] The failure of leucocytes to migrate into infected alveoli prevents phagocytosis of the bacteria and thus interferes with the natural defenses of the lung.[21] Which, if any, of these processes are directly influenced by cortisone is at present not known. It is clear that cortisone (a) does not enhance bacterial growth per se, (b) does not decrease the absolute number of circulating neutrophils, and (c) does not affect phagocytosis once exudation of leucocytes into the alveoli has occurred.

The earlier occurrence of bacteremia in the cortisone-treated animals with pneumonia may be due to a single factor or a combination. Increased bacterial growth at the original infection, delayed phagocytosis within the alveoli, and failure of the inflammatory defenses of the regional lymph nodes may all be operative; on the basis of the evidence at hand, no statement regarding their relative importance is justified.

It is of interest that in the bacteremia experiment, where a small inoculum was used to produce a less fulminating infection, the cortisone-treated animals appeared less toxic than the controls for many hours; nevertheless, in the end, they died more promptly from the infection. This "symptom-masking" effect of cortisone appears to be analogous to that reported by others in human patients.[22, 23, 27]

SUMMARY

Cortisone shortens the survival time of mice with cervical adenitis due to group A streptococci. Rats with streptococcal pneumonia were similarly affected by cortisone therapy. The pneumonic lesions in cortisone-treated animals exhibited the following differential features: (a) the edema response was accentuated, (b) the bacterial population of the infected alveoli was often excessive, and (c) the migration of neutrophilic leucocytes into the lesions appeared to be depressed. Although individual variations in these findings were observed, the general differences were relatively consistent. The unfavorable effect of Cortisone upon the infection, including the precipitation of early bacteremia, cannot be attributed either to enhancement of streptococcal growth per se or to a decrease in the number of circulating polymorphonuclear leucocytes. Systematic histologic studies suggest that the detrimental action of Cortisone in acute bacterial disease may be related to an interference with the mobilization of phagocytic cells at the site of infection.

The advice of Dr. William H. Daughaday in the planning of the preliminary aspects of this investigation was most helpful.

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CHRONIC MELIOIDOSIS: BACTERIOLOGIC AND CLINICAL CORRELATION IN DIAGNOSIS*

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THE occurrence of melioidosis, a glanders-like disease, originating in the Western Hemisphere, is quite rare. McDowell and Varney[1] in 1947 reported the first case of chronic melioidosis believed to have been contracted within these geographical boundaries. One year later Beamer[2] added another case observed in a woman member of the United States Marine Corps who had not been out of the United States except for two years in Panama, several years prior to the onset of the disease.

In 1910 Whitmore and Krishnaswami[3] observed the first cases of melioidosis in Rangoon beggars at postmortem, and their report was published in 1912 along with a description of the etiologic agent. Since then over 300 cases have been recorded in the medical literature, almost all occurring in the endemic area of the Far East. The causative organism, *Malleomyces pseudomallei,* initially described by Whitmore, is a small, pleomorphic, rod-shaped, gram-negative bacillus. Usually, it produces a glanders-like disease which is fatal in about 95 per cent of cases within a period of one month. Clinical varieties which have been observed fall into two main groups: the acute or subacute septicemic type comprising the great majority of cases and usually terminating fatally, and the chronic type, of which only seven such cases have previously been reported and only three, including this case, have been indigenous to the Western Hemisphere.

In the acute form, the onset is sudden with malaise, moderate to high fever, signs of acute pulmonary infection, septicemia with widespread visceral involvement, prostration, and death in a few days. The chronic type presents, to a surprising degree, the clinical characteristics of the more common mycoses with evidence of multiple small discrete and large confluent abscesses of the skin, viscera, and bones, with sinus formation.

Mode of transmission is not known, though in the Far East it is believed to be a disease of rodents, and dissemination supposedly results from contamination of foods with the excreta of rats. In 1941 Blanc and Baltazard[4] demonstrated experimental transmission by the *Aedes aegypti* mosquito and the rat flea, *Xenopsylla cheopsis.* Human beings do not appear to be readily infected, as may be deduced from its rarity even in endemic areas where the inhabitants are exposed to a huge rat population in an environment of filth, malnutrition, and squalor.

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There is no specific treatment for the disease, though Grant and Barwell[5] have reported successful use of various sulfonamide preparations in a chronic case. The known tendency for spontaneous remissions in individuals affected with the chronic type of the disease makes evaluation of therapy extremely difficult. The antibiotic, penicillin, and autogenous vaccines have been used without success. Newer antibiotics are worth trial in similar cases providing sensitivity of the organism to reasonable concentrations of the drug in vitro can be demonstrated. Surgical treatment is indicated for early incision and drainage of abscesses.

A case of chronic melioidosis is presented with clinical and bacteriological observations; bacteriologic and pathologic findings noted in experimentally infected guinea pigs are described.

CASE REPORT

History.—The patient, C. H. S., a 45-year-old white man, was admitted to the Veterans Administration Hospital on March 9, 1950, with complaints of nausea and vomiting, substernal pain, and a progressively enlarging, red, hot, tender area on the inner aspect of the right thigh. His present illness began about midnight of March 3, when he awoke to find that he had fallen out of bed and was violently ill. Previous to retiring he had drunk "several" bottles of beer and had eaten some fresh halibut.

He stated that he had been in the Army from October, 1942, to January, 1943, but had never left the continental limits of the United States. He admitted to excessive drinking until three years ago when he took the "cure" and he had drunk only moderately since. He had been variously employed at many occupations, but between 1943 and 1947 he worked as a hand in the Chicago stockyards. While at this latter occupation in 1943 he suffered a crushing injury of the third and fourth fingers of the left hand, and he developed a wound infection which drained for ten days; since then he had noted intermittent swelling and pain in the third finger, left hand, without abscess formation or drainage. Eighteen months ago, following trauma, his right heel became infected, with abscess formation and drainage which lasted for fourteen days. Careful questioning elicited a possible exposure to rats while living in a Chicago "flop" house.

Physical Examination.—On admission the patient appeared obviously jaundiced and acutely ill, though he was able to walk with considerable difficulty due to the pain in his right thigh. The temperature was 101.8° F.; pulse 94; respirations 20. The mucous membrane of the mouth was pale, and oral hygiene poor. The chest examination was essentially negative. The abdomen was slightly distended and markedly tender, with maximum tenderness over the entire right side; the superficial venous pattern was prominent over the right side of the abdomen and the right lower thorax, anteriorly; the liver edge was palpable 4 to 5 finger-breadths below the right costal margin and tender. On the anteromedial aspect of the right thigh, extending caudad from the inguinal ligament, was a large erythematous area measuring 30 by 20 cm.; the central portion, which covered most of the lesion, appeared a deep purple-red color, was hot, exquisitely tender, and elevated; the margin of the affected area, which measured 2.5 cm., was slightly less elevated and of a yellow-red color. Above the inguinal ligament, and parallel to it, was a smaller lesion similar to the one described.

Laboratory Examination.—The red cell count was 3,200,000, and the hemoglobin was 11 Gm. on admission, but a moderate secondary anemia developed which required repeated small whole blood transfusions. The white blood count varied from 5,400 to 16,000, with a polymorphonuclear leucocytosis and stab forms ranging from 10 to 28 per cent. Erythrocyte sedimentation rate (Westergren) was 42 mm. per hour. A red blood cell fragility test showed hemolysis starting at 0.42 and complete at 0.18. Serum proteins measured 5.59 Gm. with an albumin globulin ratio of 1.04, which later reversed to 0.54, then gradually returned to 1.58. Nonprotein nitrogen was 24 mg. per 100 milliliters. Urinalysis showed a slight trace

of albumin, and a positive reaction for bile. Liver function tests showed evidence of extensive liver involvement; serum bilirubin 3.5 mg. per cent at one minute—5.0 mg. per cent at twenty-five minutes; thymol turbidity 10.7 units; cephalin cholesterol flocculation 4 plus; total cholesterol 230 mg. per cent; cholesterol esters 45 mg. per cent; Kahn and Wassermann were negative. *E. typhosus O*—positive 1:88; *S. paratyphi*—positive 1:80; *S. Schottmulleri*—positive 1:16; *Brucella melitensis*—negative; Weil-Felix reaction—negative; *B. tularensis*—negative. Blood culture on March 21, 1950—no growth.

X-ray examination of the chest, pelvis, and femurs was essentially negative. A flat plate of the abdomen revealed a soft tissue density in the right upper quadrant producing a slight pressure defect on the hepatic flexure of the colon; this was originally considered compatible with a distended gall bladder; the liver edge was visualized at the level of the crest of the ileum posteriorly, and there was a minimum degree of small bowel ileus on the left side; several calcified deposits were noted in the spleen, but there was no splenic enlargement. The gall bladder series revealed a normally functioning organ with no evidence of stones.

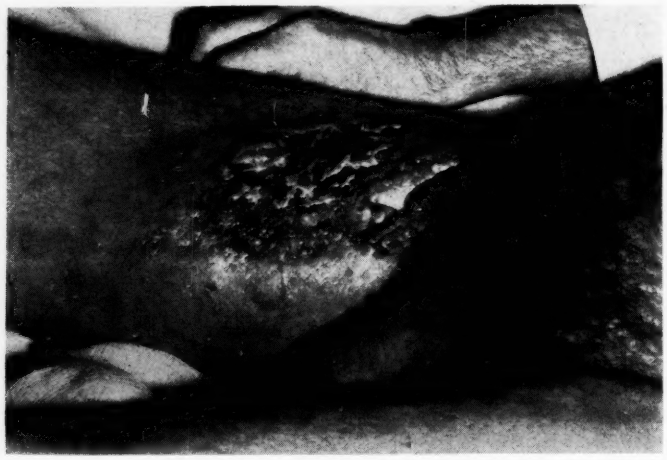

Fig. 1.

Hospital Course.—During the first four days the patient was unable to tolerate anything by mouth since it would induce symptoms of partial bowel obstruction. The abdominal signs slowly diminished until, on March 13, the abdomen became soft, flat, and nontender except for the area of cellulitis. However, he continued to appear very sallow and toxic. The affected area over the right lower abdomen and right thigh was treated locally with hot moist magnesium sulfate compresses, and the patient was given penicillin 200,000 units intramuscularly every twelve hours and 0.5 Gm. streptomycin every six hours for three days, then twice daily for two days. On March 13, the area of erythema and edema in the right lower quadrant had increased in size and extended mesiad involving the base of the penis and the right side of the scrotum. Large blebs containing a reddish-brown fluid formed on the lesion on the right thigh. The entire central portion of the thigh lesion broke down exposing a raw, granulating surface covered with purulent exudate (Fig. 1). The patient was then placed on tyrothricin packs to the affected area, and aureomycin was administered in doses of 500 mg. every four hours. The patient continued to have a daily spiking temperature to 101° F. until March 21. On March 22, a punched-out draining ulcer was noted on the posterior aspect of the scrotum near the median raphe; five days later a new sinus was observed just to the right of the anus. The lesion on the right thigh gradually became less purulent, and healing seemed apparent (Fig. 2). On March 23, clinical jaundice was no longer evident, and repeat liver function tests showed marked improvement. The lesions continued to heal, the scrotal and pararectal sinuses closed, the patient felt well and gained weight, and was discharged from the hospital on May 26, 1950.

Bacteriologic Studies.—The microorganisms isolated in pure culture, from the serous exudate of the fulminating, inguinal lesion of the patient, from a caseous lymph node in the tunica vaginalis, and from the heart's blood of experimentally infected male guinea pigs showed the following morphologic, tinctorial, and biochemical characteristics.

Morphology: In gram-stained preparations of twenty-four hour infusion agar cultures the microorganisms varied in size from approximately 0.3 to 0.5 by 0.7 microns. They were gram-negative, non-acid fast, bipolar staining rods with rounded ends and exhibited parallel arrangement. In stained preparations of forty-eight hour broth cultures the bacilli appeared considerably longer and showed marked irregular staining, especially when stained with methylene blue.

Fig. 2.

Motility: Active; 1 to 2 flagella at one pole. (Flagella stain, Leiferson's, B.B.L.)

Spores: Absent.

Capsules: Not present in India ink preparations.

Biochemical characteristics: Growth was rapid and luxuriant in all media, both aerobically and anaerobically.

Infusion agar plate: Twenty-four hours, 37° C. Colonies round, convex, mucoid, opaque, and slightly cream colored; edges irregular. Forty-eight hours, 37° C., colonies showed heaped-up centers with translucent, striated peripheries. Cultures incubated for one week at 37° C. and one month at room temperature showed no pigment production. Six months after primary isolation, subcultures being made monthly on infusion agar slants, a greenish-blue, chloroform soluble pigment was produced.

Blood (human) agar plates: Colonial appearance the same as on infusion agar plates. Beta hemolysis was produced after forty-eight hours' incubation at 37° C.

Eosin Methylin Blue plates: Colonies were of medium size, convex, semitranslucent and colorless. After three days' incubation at 37° C., the colonies had a slight bluish color.

3 per cent glycerol agar: Colonies were smooth and mucoid, with a glistening surface. Bluish-green pigment diffused out into the medium. On further incubation only smooth colonies were observed, the rough type of colony, characteristic of *Malleomyces pseudomallei* was not produced.

Gelatin stab: Stratified liquefaction, complete in five days at 37° C.

Loeffler's serum: Confluent, raised, creamy growth. Some liquefaction after five days' incubation at 37° C.

Infusion agar slant: Twenty-four hours, 37° C. Abundant, confluent, raised, glistening growth. After forty-eight hours, brownish-pink discoloration.

Infusion broth: Twenty-four hours, 37° C. Abundant growth, even turbidity, surface pellicle. Heavy mucoid sediment after seven days' incubation. No pigment.

Dextrose: (C.T.A.—B.B.L.): Acid, surface pellicle. Six months later only slight acid production. No gas.

Lactose (C.T.A.—B.B.L.): Acid, no gas, surface pellicle. Six months later, no acidity.

Sucrose (C.T.A.—B.B.L.): Acid, no gas. Surface pellicle. Six months later, no acidity.

Mannitol: Slight acidity, surface pellicle.

Xylose: Negative, surface pellicle.

Indol: Negative.

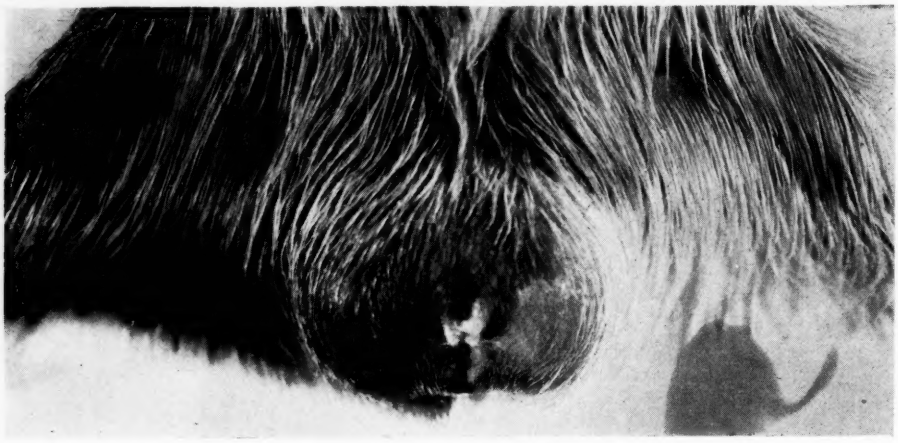

Fig. 3.

Nitrates: Not reduced.

Hydrogen sulfide: Positive.

Methyl red: Negative.

Vogues-Proskauer: Negative.

Brom-cresol-purple milk: Complete peptonization in five days, slight alkalinity.

Catalase: Positive.

In Vitro Sensitivity Tests.—

Serial Dilution Method:

Aureomycin: resistant to 10 μg per milliliter
Chloromycetin: resistant to 125 μg per milliliter
Terramycin: sensitive to 7.8 μg per milliliter
Penicillin: resistant to 20 units per milliliter
Streptomycin: resistant to 125 μg per milliliter
Sodium sulfadiazine: resistant to 5 μg per milliliter

Difco Sensitivity Method:

Chloromycetin: resistant to 60 μg per milliliter.
Terramycin: sensitive to 10 μg per milliliter
Streptomycin: resistant to 60 μg per milliliter

Animal Inoculation.—An adult male guinea pig was injected intraperitoneally with 0.1 ml. of the serous exudate from the patient's wound. Forty-eight hours later the guinea pig developed a typical Straus reaction (Figs. 3 and 4). Seventy-two hours after inoculation the pig was sacrificed. At autopsy the lungs appeared heavy and mottled; the liver enlarged and friable; and the spleen slightly enlarged and firm. The testes were enlarged and showed extensive areas of suppuration involving the tunica vaginalis. The tunica vaginalis was inflamed and scattered with 2 to 3 mm. sized nodules filled with pus. Gram stained smears of the pus showed many polymorphonuclear leucocytes and intra- and extracellular, bipolar staining gram-negative rods, appearing to be surrounded by capsules, characteristic of *Malleomyces pseudomallei.* A pure culture of the microorganism was isolated from a nodule and had the same biochemical characteristics as the strain isolated from the patient's wound.

The histopathologic changes were as follows: lungs showed severe congestion and bronchiolitis; liver showed severe congestion; spleen showed severe congestion and an increase in eosinophiles, and the testes showed extensive abscess formation in the tunica vaginalis with an increase in the number of eosinophiles and moderate necrosis of the surrounding tissues.

Fig. 4.

Two male guinea pigs were inoculated intraperitoneally with a twenty-four hour broth culture of the microorganism isolated from the patient's lesion. The first pig, inoculated as described above, showed a positive Straus reaction in forty-eight hours. The second pig, inoculated intraperitoneally with 0.5 mg. of the broth culture, died of an overwhelming septicemia twenty-two hours after inoculation. At autopsy there was a diffuse peritonitis, splenitis, and the serosal membranes were inflamed. The abdomen contained approximately 20 ml. of a thick, mucoid pus. The tunica vaginalis was inflamed. The thorax showed acute pleuritis and pericarditis. The heart was in diastole and engorged.

Gram stained and methylene blue stained smears of the peritoneal exudate and the heart's blood of the two pigs inoculated with the twenty-four hour broth culture of the organism showed morphologically typical *Malleomyces pseudomallei.* The microorganism was recovered, in pure culture, from the heart's blood and showed the same biochemical characteristics as the original strain.

Male guinea pigs inoculated in the same manner and with the same dosage of a twenty-four hour broth culture of *Pseudomonas aeruginosa* showed no Straus reaction or signs of a general septicemia.

TABLE I. TINCTORIAL, MORPHOLOGICAL, BIOCHEMICAL AND PATHOLOGICAL CHARACTERISTICS OF MALLEOMYCES PSEUDOMALLEI, PSEUDOMONAS AERUGINOSA, AND MICROORGANISM ISOLATED FROM PATIENT'S WOUND

MICROORGANISM	GRAM STAIN	MOTILITY	PIGMENT AGAR	GLYCEROL AGAR	GELATIN	DEXTROSE	LACTOSE	MANNITOL	XYLOSE	INDOL	NITRATE	H_2S	REACTION STRAUS
M. Pseudomallei (Topley and Wilson)	0	+	0	0	+ 5 d.	+	+	+	0	0	+	+	+
M. Pseudomallei (Blanc)	0	+	0	+		+	+	+		0	0	+	+
Pseudomonas aeruginosa (Topley and Wilson)	0	+	+	+	+ 2 w.	+	0	0	0	0	0	0	0
Microorganism from patient	0	+	0	+	+ 5 d.	+	+	+	0	0	0	+	+

Note: According to Topley and Wilson, *Pseudomonas aeruginosa* will reduce nitrates if culture is tested after eighteen to twenty-four hours' incubation at 37° C.

Pseudomonas aeruginosa does not grow anaerobically, the microorganism isolated in our laboratory grew both aerobically and anaerobically.

DISCUSSION

Several features of this case point up the need for close clinical and laboratory correlation as a prerequisite for diagnosis. In our opinion this may best be accomplished by a consideration of bacteriologic and pathologic evidence followed by diagnostic differentiation of the medical problem.

Laboratory.—The microorganism isolated from the patient's lesion and from experimentally infected guinea pigs resembled *Malleomyces pseudomallei* morphologically, tinctorially, and in its pathogenicity for guinea pigs. Biochemically, certain characteristics ascribed to *Pseudomonas aeruginosa* while others ascribed to *Malleomyces pseudomallei* (Table I).

On primary isolation dextrose, lactose, sucrose, and mannitol were fermented with the production of acid, however, this property was lost, with the exception of the fermentation of dextrose, after six months of subculturing on infusion agar. Hydrogen sulfide was positive and gelatin was liquefied rather rapidly. The above characteristics do not apply to *Pseudomonas aeruginosa* (dextrose is sometimes fermented with a slight production of acid), but ascribe to the biochemical characteristics of *Malleomyces pseudomallei.*

Freshly isolated strains produced pyocyanin only when grown on 3 per cent glycerol agar, whereas it is typical of most strains of *Pseudomonas aeruginosa* to produce the pigment when grown on infusion agar or in infusion broth.

In pathogenicity the organism resembles *Malleomyces pseudomallei* producing a positive Straus reaction[6] in male guinea pigs, forty-eight hours after intraperitoneal inoculation.

The microorganism also differs from *Pseudomonas aeruginosa* in its in vitro sensitivity to terramycin, the former being very sensitive (within clinical levels) and the latter being very resistant.

It is pointed out by Topley and Wilson[7] that *Malleomyces pseudomallei* is tentatively assigned to the genus *Malleomyces,* but should that genus obtain a

permanent place in systematic bacteriology it is by no means certain that it will be placed within it.

Some investigators, Le Groux and Genevary,[8] and LeGroux and DJemil[9] consider *Malleomyces pseudomallei* to be more closely related to the genus *Pseudomonas*. Blanc, Delage, and Martin[10] reported the isolation of strains of *Malleomyces pseudomallei* which produced pyocyanin when grown on glycerol agar, but not when grown on infusion agar. Their basis of classification was on the ability of the strains to agglutinate antiwhitmori serum to a titer of 1:6400, whereas strains of *Pseudomonas aeruginosa* titered only to 1:50 to 1:400.

The strains isolated in this laboratory closely resemble the strains of Blanc and his associates. Both produced acid in dextrose and sucrose; both failed to produce indol or reduce nitrates (Topley and Wilson list *Malleomyces pseudomallei* as reducing nitrates); both produced pyocyanin only when grown on glycerol agar, for at least six months after primary isolation, and neither was able to obtain colonies of the rough type.

Unfortunately we were not able to obtain any antiwhitmori serum for serological studies.

If one accepts the above correlation plus the high virulence of the microorganism for man and guinea pigs, as criteria for identification, then the microorganism may be considered to be *Malleomyces pseudomallei.*

Clinical.—Our primary diagnostic concern was the elucidation of an infecting agent which was producing a clinical picture of rather severe intoxication. This systemic reaction was apparently being borne in greatest measure by the liver as evidenced by the jaundice, hepatomegaly and altered liver function tests. Depression of the hematopoietic system was moderate in character but findings of granulocytosis, a Schilling index of 1:7.6 and the appearance of toxic granulations indicated the infective nature of the disorder. Involvement of the skin by an erythematous lesion over the anteromesial aspect of the thigh, the inguinal area and the right side of the scrotum suggested the possibility of a cellulitis due to one of the more common pathogens. Subsequent dissolution of the skin of the involved areas with abscess and sinus formation and granulomatous response confused the issue still further and demanded consideration of the possibility of mycotic infection. At this time, bacteriologic studies indicated the presence of an unusual infecting organism, tentatively identified as *Malleomyces pseudomallei,* which seemed to clarify the clinical aspects and allowed the diagnosis of chronic melioidosis.

However, the organism failed to subscribe in every detail to bacteriologic criteria established for positive identification of *Malleomyces pseudomallei.* This necessitated close clinical-laboratory correlation in order to exclude *Pseudomonas aeruginosa* as a possible etiologic agent. Further bacteriologic data accumulated over several months seemed to implicate the *Malleomyces* even more strongly, but completely irrefutable evidence was not obtainable from this source.

As has been previously stated, very few cases of chronic melioidosis have been described in the literature. The granulomatous skin lesions with discrete and confluent abscesses, the evidence of modified intoxication with hepatitis,

hypochromic anemia, low-grade fever and albuminuria, and identification of the infecting agent seem to secure the diagnosis in this patient. In contradistinction, among the prominent signs of sepsis due to pseudomonas, are the cutaneous lesions which start as macules and vesicles, progress through a bullous and often pustular stage, and terminate as sloughing areas on a gangrenous base. Next in frequency to the skin involvement is implication of the gastrointestinal tract; this is ulcerative in nature and may occur anywhere in the alimentary system. The clinical picture in pseudomonas septicemia is not unlike that produced by many other types of organisms; there are high fever, chills, prostration, and embolic phenomena in various organs.[11] In chronic melioidosis the course is milder, the respiratory system is involved next in frequency to the cutaneous system, the gastrointestinal tract is rarely implicated, and the liver has demonstrated invasion in about 10 per cent of cases. Finally the organisms isolated from our patient's wound satisfied the bacteriologic criteria presented by Blanc and associates, and was comparable in every way save one to the *Malleomyces pseudomallei* of Topley and Wilson.

SUMMARY AND CONCLUSIONS

1. A case of chronic melioidosis is presented which demonstrated some of the features similar to those found in *Pseudomonas aeruginosa* infections.

2. The diagnosis was established by close clinical and laboratory correlation.

3. Aureomycin 0.5 Gm. every four hours by mouth and Tyrothricin packs continuously applied to the cutaneous affection appeared to effect a favorable response with disappearance of systemic reaction and healing of the skin lesions.

4. This patient represents, so far as we are able to ascertain, the first case of chronic melioidosis completely indigenous to continental United States.

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COMPARATIVE IN VITRO STUDY OF GUINEA PIG BRAIN OXIDATIONS AS INFLUENCED BY XENON* AND NITROUS OXIDE

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DURING the past five years Lawrence and associates,[1] Lazarew and coworkers,[2] Tobias and associates,[3] and Cullen and Gross[4] have reported observations pertaining to the narcotic effects of the inert gas xenon on animals and human beings. The four operative procedures performed on human patients during xenon anesthesia at the State University of Iowa Hospitals include an orchidectomy, an evaluative celiotomy, an abdominal tubal ligation, and a dilatation and curettage and LaForte operation. A brief description of some of these clinical experiences has been reported.[4] Thus it appears that xenon is an undoubted anesthetic agent in concentrations of about 80 per cent with oxygen. In view of this fact and the inertness of the gas, it seemed advisable to begin a series of studies, of which this paper is the first, in order to learn more about the pharmacologic activity of xenon and to serve as a basis for the direction of additional investigations on the mechanism of xenon anesthesia.

This paper deals with the influence of xenon-oxygen and nitrous oxide-oxygen mixtures on the in vitro oxidations of guinea pig brain tissue. Nitrous oxide was selected as a comparative gas for several reasons: (a) it is a relatively inert inorganic gas; (b) the depression of in vitro brain oxidation by nitrous oxide reported by Quastel and Wheatley,[5] though not substantiated by Bulow and Holmes[6] and Bulow,[7] continues to be quoted; and (c) a literature search revealed no evidence of a critical study using a satisfactory statistical method for evaluating the influence of nitrous oxide on in vitro oxidations of brain with gas mixtures acceptable in current practices of anesthesiology.

MATERIALS AND METHODS

Technique.—The experiments utilized standard manometric procedures,[8] and provided duplicate analyses for each of three gas mixtures, with and without the addition of glucose as substrate, for each of five guinea pig brain specimens, and for each of eight thirty-minute periods—a total of 480 observations. The gas mixtures were Xe-O_2, N_2O-O_2, and N_2-O_2 (air), all in 80 to 20 per cent mixtures. The Xe-O_2 mixture was prepared by the water displacement technique and the N_2O-O_2 mixture was taken directly from a Foregger anesthesia machine. Each mixture was placed in a rubber bag and used to displace the air in the appropriate vessels through manifold arrangements so that all flasks receiving a particular mixture were gassed simultaneously. Experiences with a Pauling oxygen meter used in a pre-

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liminary study aimed at saving the expensive xenon indicated that a gassing period of one minute at 3 to 4 cm. water pressure was sufficient for adequate replacement of gases in the vessels. Actually, a two-minute period was used in these experiments.

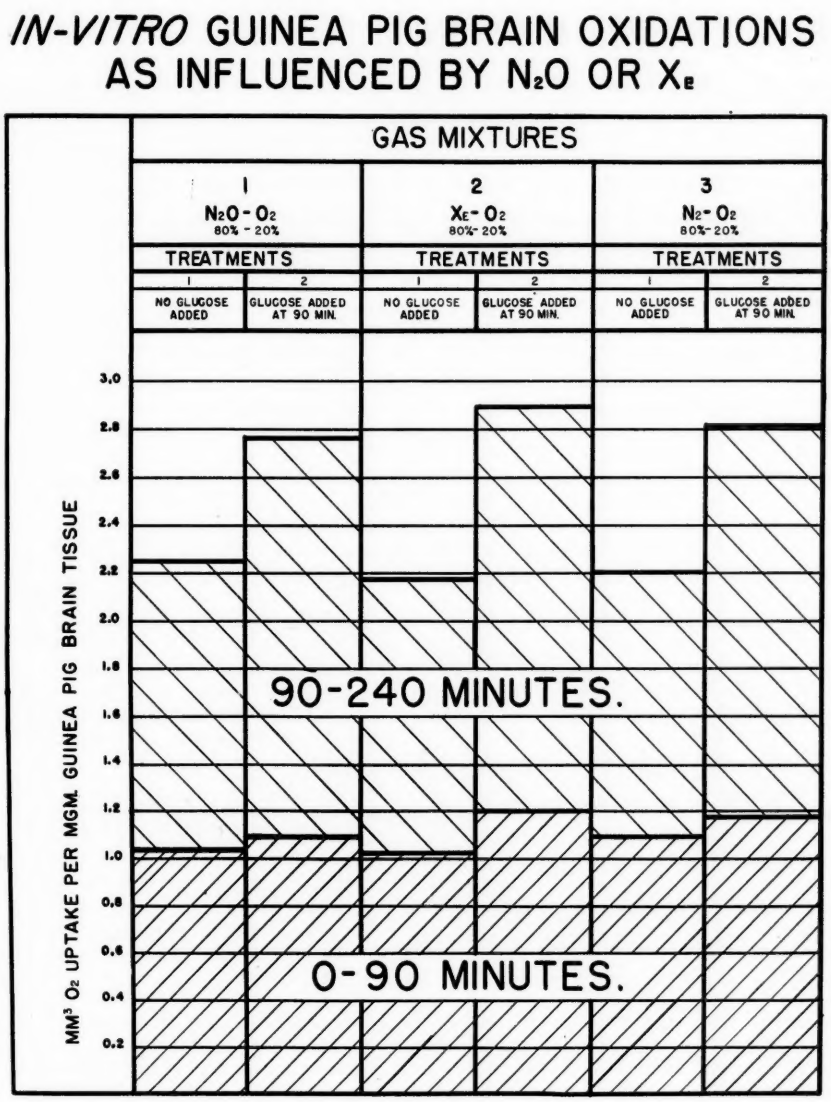

Fig. 1.

Approximately 100 mg. of the hashed cerebral hemispheres of the freshly killed guinea pig were used for each vessel. Glucose in a final concentration of 0.02M was added following the first ninety minutes of the observation period in half the vessels. Situations were duplicated on opposite sides of the bath but were randomized among the positions on a side. All solutions used in the vessels were saturated with the appropriate gas mixtures prior to the addition of the brain tissues.

RESULTS AND DISCUSSION

Data based on means of duplicate analyses of the five guinea pig brain specimens subjected to two treatments for each of the three gas mixtures are shown graphically in Fig. 1. A summary of the analysis of variance[9] obtained in the statistical treatment of the experimental data is represented in Table I. Although Fig. 1 shows no appreciable differences in the oxygen uptakes among the three gas mixtures, the significance of the minor variations evident cannot be ascertained without reference to the statistical summary in Table I. Gases, guinea pigs, and treatments were the three main sources of variation. The fact that the F value for "gases" was less than unity indicates that there were no significant differences among the effects of the three gas mixtures. Significant variances for animals at the 5 per cent level and for treatments at the 1 per cent level are not pertinent to the major question. Data for individual periods and the development of the proper error term for period variations have not been represented in Table I, since such variations were not of primary importance, and followed approximately the same course as noted in earlier experiments.[10, 11]

TABLE I. SUMMARY OF ANALYSIS OF VARIANCE FOR SUBSTRATE AND NO-SUBSTRATE TREATMENTS APPLIED WITH N_2O-O_2, Xe-O_2, AND N_2-O_2 ON CORTEX OF GUINEA PIGS 1-5 THROUGH PERIODS 1-8

	D.F.	SUM OF SQUARES	MEAN SQUARE	F	SIGNIFICANT F AT LEVEL OF 1%†	5%*
Animals (A)	4	0.334321	0.083580	5.0812*	7.01	3.84
Gases (G)	2	0.000621	0.000311	0.0189		
N_2O and Xe	(1)			<1.0		
N_2O and air	(1)			<1.0		
Xe and air	(1)			<1.0		
Error A						
A × G	8	0.131598	0.016449			
Treatments (T)	1	0.711635	0.711635	18.1655†	9.33	4.75
G × T	2	0.013946	0.006973			
Samples (S)	30	0.011841	0.000394‡			
Error B	12	0.470106	0.039175			
A × T		0.104563				
A × G × T		0.365543				
Periods and period interactions	420					
Total	479					

Gas Mixtures
1 80% N_2O — 20% O_2
2 80% Xe — 20% O_2
3 80% N_2 — 20% O_2

Treatments
1 Without glucose
2 With glucose

*Significant F at 5 per cent level.
†Significant F at 1 per cent level.
‡*Samples* M.S. < *Error B* M.S.: Indicates duplicate samples provided sufficient accuracy.

The conclusions that may be drawn are that, in these experiments, (a) no significant differences existed among the effects of xenon-oxygen, nitrous oxide-oxygen, or nitrogen-oxygen on in vitro oxidations of guinea pig brain during an observation period of four hours when no substrate was added; and (b) the increased oxygen consumption following the addition of glucose as substrate was not significantly different among the three gas mixtures.

Lack of evidence of depression by nitrous oxide of oxygen uptake by brain tissue in vitro, either with or without added substrate (glucose), is in accord with the findings of Bulow and Holmes, and disagrees with those of Quastel who reported a depression of oxygen uptake in the presence of added glucose, but not prior to addition of the substrate. It should be emphasized, however, that no attempt was made to duplicate the sample size and low oxygen concentrations used by Quastel. The present data suggest that further studies of the mechanism of xenon anesthesia might well be made in directions other than along oxidative lines.

SUMMARY

An experiment was devised for the determination of the comparative influences of Xe-O_2, N_2O-O_2 and N_2-O_2 gas mixtures, all in the ratio of 80:20, on the oxygen consumption of guinea pig brain tissues in vitro. The data indicated the following: (a) Xe and N_2O in 80:20 mixtures with oxygen did not suppress in vitro oxygen uptake of guinea pig brain tissue, either before or after addition of glucose as substrate; and (b) addition of substrate resulted in significant increases in oxygen consumption, but no significant differences existed among 80:20 Xe-O_2, N_2O-O_2 and N_2-O_2 mixtures in this respect.

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THE INFLUENCE OF OXYGEN AND CARBON DIOXIDE LEVELS DURING ANESTHESIA UPON POSTSURGICAL HEPATIC DAMAGE

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WORKING with members of the University of Wisconsin Department of Anesthesiology, Pohle[1] found that the use of chloroform resulted in postsurgical hepatic damage no more frequently than did the use of other common anesthetic agents. Reason for this divergence from general experience was sought in the conditions of his study. The type of operation and physical condition of his patients not being unusual, interest was directed to the fact that the anesthesia had been administered by highly trained physicians who gave special attention to maintenance of adequate gaseous exchange and a high concentration of oxygen in the anesthetic atmosphere. The present study was designed to evaluate the influence of these factors upon the frequency of hepatic damage.

PROCEDURE

The patients followed had been studied carefully on the medical wards of the Wisconsin General Hospital before referral for operation. The limited number of patients available for study precluded a large series subjected to a single type of surgical procedure. In general, all consecutively available cases were included in the study. Patients with biliary obstruction or any evidence of pre-existing hepatic damage were excluded, as, for reasons of safety and convenience, were those considered very poor operative risks. A total of eighty-six patients with eighty-eight anesthetic administrations were followed. In each case, a series of liver function tests were performed preoperatively. It was repeated as soon as practicable postoperatively, usually at the end of the first twenty-four hours, and thereafter at forty-eight-hour intervals until five groups of tests had been performed postoperatively, or until all tests had returned to normal, whichever represented the longer period. To insure comparable data, all tests were carried out by the methods previously utilized by Pohle,[1] and the same standards of abnormality were accepted. These included the icterus index (ten units or more), cephalin cholesterol flocculation (2 plus or more), a 2 mg. per kilogram twenty-minute bromsulfalein excretion test (5 per cent or more retention), plasma prothrombin (70 per cent or less), and thymol turbidity (2 units or more, utilizing the Kingsbury visual standards). Values just exceeding the limits of normal in one or two tests were considered as indicative of mild impairment of hepatic function; more marked changes in a single test or slightly abnormal values in three or more tests were interpreted as indicative of well-developed hepatic damage. Postoperative thymol turbidity levels exceeded 4 units in only seven patients. In only one instance was a thymol turbidity of 2 to 4 units found as the only evidence of hepatic dysfunction. This patient, who was in the chloroform low oxygen group, had a single thymol turbidity reading of 3 units on the second postoperative day, all other tests being normal. He was arbitrarily classed as having no hepatic functional impairment.

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Choice of agent was at the discretion of the anesthetists, who were aware that the patients were subjects of study, and that roughly equal numbers of chloroform and non-chloroform cases were desired. In thirty-eight operations the patient received chloroform, in forty ether, and in the remaining ten various agents which included pentothal and curare, nitrous oxide and curare, nitrous oxide and avertin, procaine spinal, and procaine spinal combined with nitrous oxide and cyclopropane.

The gaseous content of the anesthetic atmosphere and the apparent oxygen saturation of the patients' blood were subjects of constant clinical evaluation by the anesthetists. In a considerable number of the patients chemical determinations of oxygen and carbon dioxide content of venous blood were made at selected times during the operation. The methods of Van Slyke[2] were employed. Oxygen saturation was calculated on an assumed capacity of 1.3 ml. per gram hemoglobin. Those cases classed as low oxygen ranged from 24 to 80 per cent saturation in venous blood on this basis, with the majority between 40 and 70 per cent saturated.

Four patients, each subjected to palliative surgery for incurable neoplasms, were intentionally exposed to an atmosphere containing 15 per cent or less oxygen but with normal (4 per cent or less) carbon dioxide. Patients tabulated as "high carbon dioxide, normal oxygen" were intentionally exposed to atmospheres containing 10 per cent or more of carbon dioxide, plus at least 25 per cent, and usually much higher levels of oxygen. Those in the "low oxygen, high carbon dioxide" category were all patients in whom the anesthetist, in spite of his best efforts, was unable, due to the type of operation or various cardiac and respiratory complications, to maintain adequate respiratory exchange. Cyanosis was present in all of this last group, and in a majority the anesthetist's clinical evaluation was supplemented by chemical determinations of oxygen and carbon dioxide blood levels. Periods of controlled hypoxia or hypercapnia were continued for at least twenty-five to thirty minutes. No period of uncontrolled impairment of gaseous exchange was considered adequate for purposes of this study if it lasted less than twenty minutes. Analysis of the groups of cases exposed to each combination of agent and atmospheric gas concentrations shows them quite similar as to age, sex, nutritional status, complicating incidental disease, estimated operative risk, and type of operation, except that there was a somewhat higher incidence of intrathoracic operations in the low oxygen, high carbon dioxide group.

RESULTS

Restriction of variables to a single factor would have been highly desirable in a study of this type, but since such uniformity was unfortunately not practicable, and since many of the variations in gaseous exchange were purely fortuitous, analysis of several influences was required.

The incidence of hepatic damage in the two sexes was almost identical, and plotting of the frequency and severity of functional impairment in relation to the age by decades revealed no correlation. Surprisingly the "nutritional status" had no significant effect. This is possibly dependent on the fact that the state of nutrition was evaluated on the basis of long-term preoperative food intake, total weight loss, evidences of vitamin deficiency, and similar factors. Immediately before operation and continuing afterward, every effort was made to place the patients in optimal nutritional condition by supplying vitamins, protein, parenteral glucose, etc., where needed. This protection was probably much more significant from the standpoint of susceptibility to hepatotoxic factors than was the long-term nutritional status.

Complicating preoperative cardiac and pulmonary disease did not greatly influence the incidence of postoperative hepatic damage. When pulmonary or

cardiac difficulty occurred during or immediately after operation, however, the frequency of hepatic injury increased slightly, and its severity considerably. This discrepancy is probably explained by the fact that with known preoperative difficulty every precaution was taken to prevent serious impairment of circulatory status or respiratory exchange, and uncontrolled cases were not operated upon. In the case of operative and postoperative complications there was usually an unavoidable period of either circulatory inadequacy or respiratory embarrassment, or else an infectious process which may well have abetted any other hepatotoxic factors. Such complications included marked cardiac arrhythmias, cardiac arrest, marked fall in blood pressure, respiratory obstruction, pneumonia, and other postoperative infections, and atelectasis. A total of forty such episodes was recorded in thirty-one patients in this series. Twenty-one of these patients showed well-developed damage, five mild, and five none. Of the fifty-seven patients not developing such difficulty, thirteen showed severe, twenty-six mild, and eighteen no hepatic functional impairment. In view of the frequency of such difficulties, and their probable relation to abnormalities of gaseous exchange, it did not seem justifiable to exclude them from the series. It will be seen in Table I, however, that the number of patients suffering such complications by no means paralleled the number with hepatic damage in the various groups when tabulated according to anesthetic agent and carbon dioxide and oxygen levels.

It had been expected that the duration of anesthesia would have considerable influence upon the degree of hepatic damage. Comparison of the rate of such injury with total duration of anesthesia revealed less than 10 per cent variation between any two groups when the cases were tabulated as to length of anesthetic period in multiples of one-half hour. The total duration of anesthesia did not parallel the duration of abnormality in gaseous exchange, of course, and it is probable that this latter factor was more significant.

It has previously been noted by various groups, including Keeton and his associates[3] and Engstrand and Friberg[4] that certain operations represent a greater load on the liver than others. To a certain extent this seemed to be true in the present series (Table II). The differences were not marked, however, and when figures for an individual operation were examined, in every instance the frequency and severity of hepatic functional impairment were greater when the atmosphere was "abnormal."

Table I also shows the occurrence of hepatic injury when considered with reference to anesthetic agent and levels of oxygen and carbon dioxide. Even when quite high oxygen and low carbon dioxide concentrations were maintained, a number of patients gave evidence of damage. Under these conditions variation between the effect of different agents was not great, as previously demonstrated by Pohle,[1] although in our relatively small group there was a higher incidence of damage when chloroform was employed. When the blood oxygen was maintained at saturation levels, increased carbon dioxide concentrations were associated with an increase in the frequency of liver damage. The severity of this damage was considerably greater when chloroform was

TABLE I. RELATIONSHIP OF ATMOSPHERE AND AGENT TO INCIDENCE OF LIVER DAMAGE*

		CHLOROFORM				ETHER			
		ATMOSPHERIC GASES				ATMOSPHERIC GASES			
		O_2 HIGH CO_2 LOW	O_2 HIGH CO_2 HIGH	O_2 LOW CO_2 LOW	O_2 LOW CO_2 HIGH	O_2 HIGH CO_2 LOW	O_2 HIGH CO_2 HIGH	O_2 LOW CO_2 LOW	O_2 LOW CO_2 HIGH
TOTAL CASES		10	17	4	7	29	10	0	11
Number of patients with abnormal function tests	B S P	5	12	3	5	9	5	–	7
	Ict. index	7	10	2	4	8	4	–	4
	Ceph. floc.	3	7	2	5	5	2	–	4
	Prothrombin	6	9	1	5	11	3	–	4
	Thymol	4	8	3	3	5	2	–	3
Degree of damage	+	3(30)	10(59)	3(75)	5(72)	5(17)	3(30)	–	5(46)
	±	4(40)	5(29)	0	2(28)	11(38)	6(60)	–	3(27)
	0	3(30)	2(12)	1(25)	0	13(45)	1(10)	–	3(27)
Operative and post-operative complications	Total number	2(20)	8(47)	3(75)	2(28)	3(10)	6(60)	–	7(64)
	Degree of damage +	1	6	3	2	2	3	–	4
	Degree of damage ±	0	1	0	0	0	3	–	1
	Degree of damage 0	1	1	0	0	1	0	–	2

*The incidence of complications is shown for each group. Actual numbers of cases are shown. Figures in parentheses indicate per cent of the total number subjected to that combination of agent and atmospheric gases. Agents other than chloroform are included in the other group. + indicates well developed, ± mild, and 0 no hepatic functional impairment. See text for criteria as to atmospheres, complications, and grading of damage.

the anesthetic agent. When oxygen levels were permitted to fall below saturation values and carbon dioxide concentrations to rise simultaneously, the incidence and severity of damage in those cases receiving chloroform was markedly increased, although the small number of cases renders the significance of this shift questionable. With other agents, no clear-cut interpretation could be assigned to the differences between the high carbon dioxide high oxygen and the high carbon dioxide low oxygen groups. The number of cases was too small to permit definite conclusion, but with low oxygen and normal carbon dioxide levels, the tendency to damage was approximately the same as in the high carbon dioxide groups.

In each of the three groups with abnormal gaseous exchange the incidence or severity of hepatic damage seemed, then, to be increased when compared with the high oxygen low carbon dioxide group. The use of chloroform in association with such abnormal exchange tended to increase either the frequency or degree of damage or both, but without marked difference in this respect between the three abnormal groups.

Eight patients died before release from the hospital. In none was the death directly attributable to hepatic damage, but all showed laboratory evidence of such injury, and two were visibly jaundiced. Data in regard to these patients are shown in Table III.

Table I shows the frequency with which the individual function tests became abnormal in patients subjected to varying conditions. No correlation between abnormality of any given test and a particular combination of agent and atmosphere could be demonstrated. Neither was there any predominating tendency for any two tests to become abnormal in the same patient. A rough parallel was found between abnormality of the bromsulfalein excretion and the icterus index, and between the cephalin cholesterol flocculation and the thymol turbidity, but there were frequent exceptions. In almost every instance a given test either became abnormal within forty-eight hours after operation or remained normal throughout the period of observation.

TABLE II. HEPATIC DAMAGE BY TYPE OF SURGICAL PROCEDURE*

OPERATION	SEVERITY OF DAMAGE		
	+	±	0
Cholecystectomy	5 (56)	1 (11)	3 (33)
Gastric resection	10 (50)	8 (40)	2 (10)
Thoracotomy	7 (35)	7 (35)	6 (30)
Misc. abdominal	9 (47)	6 (32)	4 (21)
Misc. nonabdominal	3 (15)	9 (45)	8 (40)

*Number of cases is shown, with per cent of total in parentheses. + represents well developed, ± mild, and 0 no hepatic damage.

DISCUSSION

A great many factors, known and unknown, are undoubtedly involved in determining the incidence and severity of postoperative hepatic damage. The significance of the more widely appreciated influences is reviewed in reports by Engstrand and Friberg,[4] Keeton and associates,[3] and Boyce and McFetridge.[5] Elimination of such variables was not possible, but when classi-

TABLE III. POSTOPERATIVE DEATHS*

AGE	TYPE OF OPERATION	DAY OF DEATH (POSTOPERATIVE)	AGENT	ATMOSPHERIC GAS LEVELS	IMMEDIATE CAUSE OF DEATH	CEPH. FLOC.	THYMOL (UNITS)	ICTERUS INDEX	PROTHROMBIN %	BROMSULFALEIN (% RETAINED)	POST MORTEM FINDINGS (LIVER)
74	Gastric resection for Ca	7	C	High CO_2 low O_2	Pneumonia and lung abscess	4	4	150	70	–	Marked centrolobular necrosis; mild diffuse parenchymatous degeneration and fatty infiltration; very mild cellular infiltration portal areas.
34	Lobectomy tuberculosis	32	E	High CO_2 low O_2	Miliary tuberculosis	3	3	5	100	60	Miliary tubercles throughout liver; moderate generalized parenchymatous degeneration and moderate round cell infiltration portal areas.
57	Closure bronchopleural fistula	8	E	High CO_2 low O_2	Massive pulmonary hemorrhage	0	2	4	59	5	No autopsy.
65	Pneumonectomy for carcinoma	6	E	High CO_2 low O_2	Pneumonia	4	2	7	–	–	Marked centrolobular parenchymatous degeneration and necrosis; moderate cellular infiltration portal areas; mild chronic passive congestion.
72	Gastric resection for Ca	10	E	High CO_2 high O_2	Pneumonia peritonitis	3	3	8	68	5	Moderate patchy centrolobular necrosis; slight diffuse parenchymatous degeneration; moderate fatty infiltration; moderate cellular infiltration portal areas.
53	Gastric resection for Ca	2	C	High CO_2 high O_2	Coronary occlusion	2	7	4	80	15	No autopsy.
73	Gastric resection for Ca	2	C	High CO_2 high O_2	Not clear†	0	0	4	59	15	No autopsy.
44	Gastric resection duodenal ulcer	10	E	High CO_2 high O_2	Hemorrhagic pancreatitis; atelectasis	2	1	25	28	35	Grossly marked parenchymatous degeneration and fatty change; no histologic specimen.

*Agents are "C," chloroform, and "E," ether and all others. Values given for liver function tests were obtained within thirty-six hours of death in each instance.

†The seventh patient listed responded very poorly after surgery, failed rapidly, and died without definitely assignable single cause.

fied according to anesthetic agent and concentrations of atmospheric gases, the various groups of patients in the present study seemed roughly comparable in regard to all other factors. If this assumption is valid, the data obtained indicate that even when supposedly optimal atmospheric gas concentrations were maintained, there was frequent impairment of hepatic function after surgical anesthesia. Either hypoxia or hypercapnia acting alone increased this damage in generally similar degree; their simultaneous occurrence did not seem to be much more injurious than that of either alone.

These results in regard to hypoxia were expected, and confirm the work of Goldschmidt, Ravdin and Lucké.[6] Indeed, Büchner[7] demonstrated in man, and Martin, Bunting and Loevenhart[8] in animals that uncomplicated oxygen lack is capable of producing centrolobular necrosis.

The effect of carbon dioxide was less predictable, although the findings of Barbour and Seevers[9] suggest that it might increase the incidence of injury. These workers found that high concentrations of carbon dioxide increased the toxicity of and prolonged the recovery time from pentobarbital anesthesia. Bromsulfalein excretion was also delayed in their animals. Their studies covered only the period of actual exposure to excess carbon dioxide, and they postulated reduced hepatic blood flow due to splanchnic vasoconstriction as the probable cause. Such a mechanism might under ordinary conditions of surgical anesthesia play a role by depriving the cells exposed to hepatotoxic agents of an adequate blood supply, with consequent hypoxia.

It does not seem probable that the tendency of high concentrations of carbon dioxide to increase the dissociation of oxyhemoglobin, which might with high atmospheric carbon dioxide interfere with oxygen uptake in the lungs, is of importance here. Henderson, Bock, Field, and Stoddard[10] have shown in vitro that with high oxygen tensions hemoglobin becomes fairly well saturated with oxygen even in spite of moderate increases in carbon dioxide tension. High oxygen concentrations were generally maintained in the anesthetic atmospheres employed in the present study, and direct measurement showed adequate oxygen levels in the purely "high carbon dioxide" cases of this study, as well as in another group of patients exposed to high carbon dioxide in the absence of any other anesthetic agent (Morris, Sims, and Orth[11]).

A direct toxic effect of the carbon dioxide itself does not seem to be important, for Morris, Sims, and Orth[11] have found that anesthetic concentrations of this agent maintained for periods of twenty-five to thirty minutes do not result in more than slight degrees of hepatic functional impairment when evaluated by the same group of tests.

Major consideration, however, must be given to the possibility that excessive carbon dioxide acts directly on the hepatic cell or its immediate environment to increase the susceptibility to hepatotoxic agents. Among the known effects of inhalation of high concentrations of carbon dioxide which might be so operative are a marked acidosis (e.g., Barbour and Seevers[9]), a considerable reduction in the oxidative processes of the body (demonstrated

by Gesell and associates[12]), and the initiation of the alarm reaction of Selye, with its chain of catabolic reactions, for which Fortier[13] has demonstrated that excessive carbon dioxide is an adequate stimulus.

In most patients subjected to surgical procedures, the degree of hepatic damage is not sufficient seriously to prejudice their recovery, nor even to be detected clinically, unless hepatic function tests are performed. In only three patients of the present series was visible icterus recorded, and in all except those who died, the hepatic function tests returned to normal within three weeks after operation. In no case was hepatic damage the direct cause of death, although in one or two it may have been a major contributing factor (Table III). The cases in the present series, it may be noted, were especially selected to exclude any with preoperative evidence of impairment of liver function, and every effort was made to attain the best practicable nutritional status preoperatively. Many patients must be operated upon in the presence of pre-existing hepatic disease, and without opportunity for extensive preoperative preparation. In such instances additional hepatic injury may represent a major hazard. The occurrence of acute postsurgical hepatic failure and death, often in the form of the so-called hepatorenal syndrome, is by no means uncommon, and the present study suggests that unrecognized or unavoidable hypoxia, hypercapnia, or both occurring during anesthesia may well be a major determining factor in certain of these deaths.

SUMMARY

1. Hepatic injury, as demonstrated by serially performed cephalin cholesterol flocculation, thymol turbidity, icterus index, prothrombin time, and bromsulfalein excretion tests, occurs in a high proportion of all patients subjected to surgical procedures, even when maintenance of supposedly optimal blood oxygen and carbon dioxide levels.

2. Hypoxia, hypercapnia, or both, considerably increases the frequency and severity of such damage.

3. With optimal concentrations of oxygen and carbon dioxide, use of either ether or chloroform gives roughly the same amount of damage. When either hypoxia, hypercapnia, or both occur, the damage is significantly greater when chloroform has been the anesthetic agent.

4. The type of operation and the occurrence of such operative or postoperative complications as marked falls in blood pressure, severe cardiac arrhythmias, and periods of respiratory obstruction also influence the occurrence of such damage, perhaps through a secondary effect upon the concentrations of oxygen and carbon dioxide in the blood.

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D-TUBOCURARINE CHLORIDE CONCENTRATIONS IN HUMAN PLASMA AFTER INTRAVENOUS INJECTION DURING ANESTHESIA*

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ACCORDING to McIntyre[1] "The fate of curare in man has not been examined and requires investigation." An important factor responsible for the absence of previous investigation has been the lack, until recently, of adequate analytical techniques for the determination of minute quantities of curare in biological materials. Quinn and Woislawski[2] reported a modification of the procedure of Brodie and Udenfriend[3] for estimating basic organic compounds which was satisfactory for the determination of d-tubocurarine chloride in concentrations as low as two micrograms per milliliter of plasma.

EXPERIMENTAL

The procedure of Quinn and Woislawski was adopted for this project. A standard curve which proved to be a straight line over the required range of values was developed from a series of solutions of varying concentrations of d-tubocurarine.

Six adult patients were used as subjects for study (Table I). Three persons were in attendance during the study of each clinical curarization. This number was essential for the anesthetization of the patient, administration of the drug, collection and labeling of the multiple samples, and maintenance of records. The patients were anesthetized and a sample of blood was drawn for determination of its blank value before curarization. The d-tubocurarine chloride was administered intravenously over a period of several seconds into one arm, and blood samples were withdrawn in heparinized syringes at appropriate times from a vein in the other arm. Since multiple samples were withdrawn an 18-gauge needle was employed and left inserted in the vein whenever possible. In order to minimize the possibility of contamination, collection of the blood samples was done by a person who had no contact with the d-tubocurarine vial or the syringe used to administer it. The initial samples for the determination of the plasma levels of the drug were drawn within one and one-half to two minutes after injection in all instances. The time of disappearance and onset of both diaphragmatic and intercostal activity was noted. During the period of apnea artificial ventilation was accomplished by the manual application of intermittent positive pressure to the rebreathing bag. Spontaneous respirations were assisted until ventilation was considered to be adequate. The blood samples of about 8 to 12 ml. each were retained in the syringes from which the needles were removed and replaced with metal caps. All samples were analyzed on the same day they were drawn.

In the laboratory 3 ml. of the plasma obtained by centrifuging the blood sample were transferred to a glass-stoppered 125 ml. Erlenmeyer flask. Ten milliliters of acid-purified ethylene dichloride and 0.5 ml. of potassium iodide-glycine buffer (pH10) were then added to the flask. After shaking and centrifuging the ethylene dichloride containing the extracted d-tubocurarine was decanted to a 15 ml. glass-stoppered centrifuge tube. Three drops of citric acid buffer (pH5) and several small particles of recrystallized methyl orange were added and the stopper seated tightly. After again shaking, centrifuging, and discarding the aqueous

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*D-Tubocurarine Chloride used in this study was supplied by E. R. Squibb & Sons.

TABLE I.

SUBJECT	1	2	3	4	5	6
Sex	F	M	F	M	F	F
Age	48	42	40	19	48	52
Weight	146	159	126	160	150	93
Anesthetic agent	C_3H_6 and Ether	C_3H_6 and Ether	N_2O and Pento	N_2O and Pento	C_3H_6	C_3H_6
Depth of surgical anesthesia	Moderate	Light	Light	Light	Light	Deep to Light
d-Tubocurarine dosage in mg.	15 @ 0847	15 @ 1111	18 @ 0845	15 @ 0745	30 @ 1103	18 @ 1109½ 9 @ 1135
Initial plasma level (micrograms)	2.8	4.7	7.5	6.0	12	13
Minutes for reduction to ½ initially determined level	2	7	7	10	5½	4½

phase a 5 ml. aliquot of the organic phase containing the d-tubocurarine-methyl orange complex was then transferred to a Klett-Summerson tube and 0.5 ml. of acid-alcohol added. The contents of the tube were shaken to produce a uniform, clear solution which was pink because of methyl orange released from the complex. Optical measurements were made after ten minutes and the values referred to the working curve to obtain the d-tubocurarine chloride content of the sample.

In the course of the project a number of experimental difficulties associated with the collection of blood samples and their analysis were encountered. The following comments may avert similar difficulties for others. Lack of specificity of the procedure and minute quantities of drug present in the plasma made the requirements for careful analytical technique imperative. Precautions during the collection of samples were quite as important as the analytical procedure to which they were subjected. Contaminants from the skin, rubber stoppers, corks, and organic materials in general had to be avoided. The syringes routinely used for drug administration in the hospital were sterile but not chemically clean and could not be used for collection of blood samples. It was necessary that all laboratory glassware be washed with soap and water, rinsed, treated with potassium dichromate-sulfuric cleaning solution, and thoroughly rinsed with tap and distilled water. All syringes and needles were likewise treated, but were then additionally rinsed with isopropyl alcohol and ether separately and allowed to dry by evaporation before autoclaving.

Occasionally sufficient hemolysis occurred to color the plasma a faint pink and ethylene dichloride a distinct yellow. This effect did not contribute to the blank as was noted several times in duplicate samples in which hemolysis was evident in one but not in the other specimen.

On repeated optical measurements of several processed specimens over a period of three weeks there was no change in value. The methyl orange color in acidified ethylene dichloride apparently is quite stable, and it is probable that colorimetric standards could be prepared for visual comparison that would retain their proper colors for prolonged periods to time.

RESULTS AND DISCUSSION

The curves (Fig. 1) indicate a typical trend of the plasma concentrations of d-tubocurarine chloride after a single injection in human beings under anesthesia. A rapid fall in plasma concentration of the drug occurs soon after injection intravenously. After fifteen to twenty minutes the plasma concentration decreases very slowly.

The average length of time required for reduction of the plasma concentrations of the drug to one-half of the initially determined levels was six minutes for the six subjects. The individual time intervals for this decrease ranged from

two to ten minutes. Although these rates of disappearance of the drug are rapid, they do not approach the findings of Marsh[4] who recently reported the disappearance of 90 per cent of doses of radioactive d-chondocurarine and d-tubocurarine dimethylether iodide within one hundred twenty seconds when administered intravenously to dogs in a quantity sufficient to produce minimal muscle paralysis.

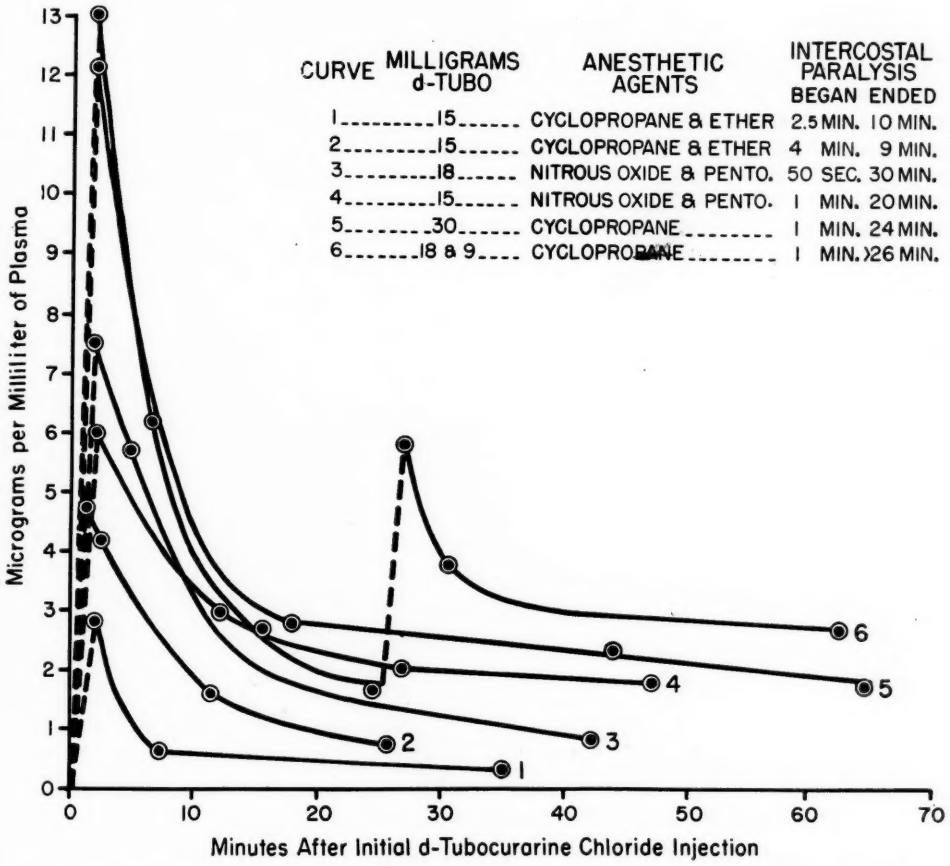

Fig. 1.—d-Tubocurarine chloride concentrations in human plasma after intravenous injection during anesthesia.

The steep slopes of the curves emphasize the importance of drawing blood samples at a specific time after injection in experiments designed to correlate plasma levels of the drug with observed pharmacologic activity. Although plasma levels were not determined on blood samples drawn later than sixty-five minutes after injection of the drug, it is apparent from the curves that d-tubocurarine chloride probably remains in the plasma for several hours thereafter. Curve 6 shows the increased plasma concentration resulting from a second intravenous injection of the drug in an amount equal to one-half of the initial dose, and also indicates a slower fall in plasma levels after the second dose than that observed after the first. This is probably due to the presence in tissue of

appreciable residual amounts of the drug from the first injection. This explanation is in agreement with the findings of Marsh,[4] who reported the presence of radioactive curare compounds in muscle and evidence of excretion therefrom long after paralysis had disappeared.

Since in each subject intercostal activity was abolished temporarily by the curare administered during anesthesia, no correlation between dosage and onset of respiratory paralysis could be made. Neither did the resumption of spontaneous intercostal activity correlate with any specific plasma level of the drug. This lack of correlation is probably attributable to the many factors, other than dosage or plasma level that affect intercostal activity, which were not controllable in this experiment. It was noted that spontaneous intercostal activity did not resume in any subject while the plasma concentration of the drug was three micrograms or greater; however, this plasma level may not represent a critical level in this respect.

Buller and Young,[5] in 1949, referred to a d-tubocurarine level of three micrograms per milliliter of circulating blood as a dose "—sufficient to inhibit respiratory movements in the urethanized rabbit rapidly and completely, but in the anesthetized human there is only slight depression of respiration accompanying the immediate muscular relaxation." These authors did not state whether the blood levels mentioned had been calculated on the basis of injected dose and blood volume, or had been determined chemically. Neither did they state how long after the intravenous injection this concentration was presumed to be present. Our observations do not concur with their statement, since complete apnea was present in five and intercostal paralysis occurred in all six subjects. It is our impression that factors such as the physical state of the patient, age, sex, premedication, depth of anesthesia, anesthetic agent, and rate of injection of the drug may be quite significant in determining the effect of the drug on respiration at *any* plasma level.

CONCLUSIONS

A study was made of d-tubocurarine chloride concentrations in plasma after intravenous injection during clinical anesthesia. Chemical determinations were made in samples of blood drawn at various times, and a typical trend of plasma concentrations was found to occur. This trend was characterized by a rapid fall in plasma concentration soon after administration, and by a very gradual fall after approximately twenty minutes. Half of the initially determined amount of the drug was found to disappear in an average time of six minutes. Extrapolation of the curves indicates the probable presence of the drug in plasma for several hours after a single injection.

There was no obvious correlation of plasma levels of the drug in the six subjects with resumption of spontaneous intercostal muscular activity after a period of paralysis. However, resumption of this function did not occur while the plasma concentrations were three micrograms per milliliter or greater.

We are indebted to Dr. E. G. Gross, Department of Pharmacology, for the generous provision of space and facilities for the laboratory portion of this study.

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THE INFLUENCE OF HEAT UPON THE RH, LEWIS, AND H AGGLUTINOGENS

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Lubinski and Portnuff[1] noticed that after a suspension of D positive red cells had been accidentally heated to 56° C. the cells were no longer agglutinated by an anti-D serum. They investigated the effect of heat further by heating cell suspensions at 56° C. for varying periods and testing their agglutinability with anti-A, anti-B, anti-M, anti-N, saline, and albumin agglutinating anti-D sera. They incubated the cell-antiserum suspensions at the appropriate temperature for one hour, centrifuged, and read the results macroscopically. They found that while heat had little effect on the A, B, M, and N antigens, the D antigen appeared to be destroyed. Hubinont[2] repeated the experiments regarding the D antigen and obtained similar results.

The aim of the present investigation has been to extend this work in addition to the above by examining those of the Rh antigens for which antisera were available and the Lewis and H antigens.

PROCEDURE

Cells.—Two per cent red cell suspensions in saline were used, the cells being freshly obtained on the day of the investigation. The cells were heated in stoppered tubes in a water bath at 55 to 56° C. The heating was continued for periods varying from twenty to forty minutes; in most of the series samples were taken at five minute intervals, in some the cells were left undisturbed and no intermediate testing done. A varying amount of hemolysis took place during the heating, which did not interfere with the readings up to thirty minutes heating. Occasionally the samples for thirty-five and forty minutes had to be discarded because of excessive hemolysis. In some series the hemoglobin-containing saline was removed after centrifuging and the heated cells resuspended in unheated saline, but no difference was noted from those series in which this was not done.

A total of 100 such samples were tested from forty-five individuals. The genotype of each person was known and their cell type confirmed against the sera being used. This procedure acted as one control for the experiments and gave a standard for the reaction of the unheated cells and sera which was necessary for judging the reaction of the heated cells. At least one cell which gave a negative reaction with the sera being used was always included in each of the series and heated at the same time as the cells under test, so that a completely comparable negative control was available for each period of heating. More than one agglutinogen was investigated in each experiment and, when a fresh agglutinogen was being tested, an agglutinogen, the effect of heat on which was known, was always included in the same experiment.

Sera.—The following sera were used: Anti-A, B, M, and N, issued by the Blood Group Reference Laboratory, London, 3 saline and 4 albumin agglutinating anti-D, 2 saline anti-C,

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2 saline anti-E, 3 albumin anti-c, 1 anti-A_1, 3 anti-Le^a, 2 anti-Le^b and 2 anti-H. One saline anti-e was used. This serum also contained anti-C, therefore no cells of phenotype C were used against this serum.

The agglutinations were all done by the tube method, the anti-A, A_1, B, M, N, H, Le^a and Le^b sera reactions being left at room temperature for one and one-half hours, and the Rh antisera reactions incubated at 37° C. for two hours. The results were read microscopically.

RESULTS

Agreement was found with Lubinski and Portnuff's work that the A, B, M, and N antigens remained and the D antigen was destroyed after heating to 56° C.

The C and E agglutinogens both behaved in the same manner as the D agglutinogen, as is seen from Table I where examples of the behavior of cells of

TABLE I. EXAMPLES OF THE BEHAVIOR OF VARIOUS ANTIGENS OF HEATING*†

CELL TYPE	SERUM	REACTION PRIOR TO HEATING	REACTION AT VARIOUS TIMES AFTER HEATING (TIMES IN MINUTES)							
			5	10	15	20	25	30	35	40
D	Anti-D	4+	3+	2+	+	(+)	W	–	–	
D	Anti-D	3+	3+	2+	(+)	–	–	–	–	–
C	Anti-C	3+	2+	+	(+)	W	–	–		
C	Anti-C	4+	3+	+	(+)	W	–	–	–	
E	Anti-E	4+	3+	3+	+	+	(+)	–	–	
E	Anti-E	4+	3+	+	+	W	–	–	–	
c	Anti-c	4+	4+	4+	4+	4+	4+	4+	4+	
c	Anti-c	4+	4+	4+	4+	4+	4+	4+	3+	3+
e	Anti-e	2+				–				
e	Anti-e	3+		(+)		W		–		
Le^a	Anti-Le^a	2+	+	(+)	W	–	–	–		
Le^a	Anti-Le^a	3+	+	(+)	–	–	–	–		
Le^b	Anti-Le^b	4+	2+	+	(+)	W	–	–		
Le^b	Anti-Le^b	3+	+	(+)	–	–	–	–		
H	Anti-H	4+	4+	4+	4+	4+	4+	4+	3+	3+
H	Anti-H	4+	4+	4+	4+	3+	3+	3+		
A	Anti-A	4+	4+	4+	4+	4+				
A_1	Anti-A_1	2+	2+	2+	2+	2+				
B	Anti-B	4+	4+	4+	4+	4+				
M	Anti-M	4+		4+		4+		3+		
N	Anti-N	4+		4+		3+		3+		

*Results expressed according to scale recommended in Mollison and associates.[3]

†The scale of results recommended by Mollison and associates[3] are as follows:

4+ Complete agglutination into one or two large clumps.
3+ Numerous clumps clearly visible to the naked eye.
2+ Granularity just visible to the naked eye; very big clumps seen under the microscope; some unagglutinated cells.
+ Not quite such big clumps; numerous unagglutinated cells.
(+) Clumps of eight to twelve cells.
W A definite but weak reaction in which there is a uniform distribution of very small clumps of about four to six cells.
– All cells separate and evenly distributed.

each group are given. On testing the effect of heat on the c antigen it was found that this was virtually unaffected by heat. The antigen e was tested but, owing to the scarcity of the serum, only ten cell suspensions could be used, and these included two controls (cDE/cDE). From this short series it appeared that the e antigen was destroyed by heat.

Both Lewis agglutinogens Le^a and Le^b behaved in a similar manner to the Rh agglutinogens C, D, and E, and examples are shown in the table.

H substance was unaffected as demonstrated by anti-H serum, the cells continuing to be agglutinated as readily as unheated cells after thirty minutes

heating. As would be expected, the anti-A_1 serum continued to agglutinate heated A_1 cells.

As the experiments proceeded it became apparent that the degree of destruction or impairment of the D antigen depended both on the length of time the cell was heated and on the cell itself, some cells becoming inagglutinable by anti-D sera more easily than others. As would be expected, the anti-D serum with the highest titer used demonstrated the agglutinogen after heating for longer periods, though a definite reduction in the degree of agglutination was always obtained. That the variation is due to the strength of serum used is shown by a strong anti-D serum being diluted in saline or AB serum, and the experiments repeated. In Table II the reactions of various heated and unheated cells are shown with an anti-D serum of titer 1:256, and with the same serum diluted 1:16.

TABLE II. THE EFFECT OF DILUTION OF AN ANTI-D SERUM (TITER 1:256)

CELL TYPES	CDe/cde	CDe/CDe	CDe/cDE	C^wDe/cde	CDe/CDe	cde/cde
Undiluted serum Unheated cells	4+	4+	4+	4+	4+	–
Undiluted serum Cells heated for 25 min.	2+	2+	+	2+	2+	–
Serum diluted 1:16 Unheated cells	4+	4+	4+	4+	4+	–
Serum diluted 1:16 Cells heated for 25 min.	–	W	–	–	–	–

This effect of dilution was used to investigate further those antigens unaffected by heat, and particularly to find whether the antigen c could be shown to be more in keeping with the other Rh antigens. Dilutions of the appropriate sera were made until a much reduced reaction was obtained with unheated cells and the heating experiments repeated. Table III shows the results obtained from diluted anti-A and anti-B serum, and confirms the thermostability of the antigens A and B. The antigen c gives results comparable with those of A and

TABLE III. THE EFFECT ON HEATING EXPERIMENTS BY DILUTING ANTI-A AND ANTI-B SERA

DILUTION	REACTION PRIOR TO HEATING	REACTION AT VARIOUS TIMES AFTER HEATING (MINUTES)						
		5	10	15	20	25	30	35
Anti-A with A cell:								
Undiluted	4+	4+	4+	4+	4+	4+	4+	4+
1:50	2+	+	(+)	+	+	+	(+)	(+)
1:100	(+)	(+)	(+)	W	W	(+)	W	W
Anti-B with B cell:								
Undiluted	4+	4+	4+	4+	4+	4+	4+	4+
1:50	2+	2+	2+	2+	+	2+	2+	+
1:100	2+	2+	+	+	+	+	+	+

B as seen in Table IV. For completeness the antisera M, N, and H were similarly treated and gave similar results (see Table IV). It can be seen from these tables that the antigens are not absolutely thermostable and that this is most pronounced with regard to the antigen c, but it is also evident by comparing

Tables II and IV that the antigen c is considerably more stable than the antigen D. By comparison the dilution experiments do not invalidate the results obtained using undiluted serum.

TABLE IV. THE EFFECT ON HEATING EXPERIMENTS BY DILUTING ANTI-c, H, M, AND N SERA*

CELL TYPE	ANTISERUM	DILUTION	REACTION PRIOR TO HEATING	REACTION AT VARIOUS TIMES AFTER HEATING (TIME IN MINUTES)						
				5	10	15	20	25	30	35
c	anti-c	Undiluted	4+	4+	4+	4+	4+	4+	4+	4+
		1:10	4+	4+	4+	2+	+	+	(+)	W
		1:20	2+	+	(+)	(+)	W	W	W	W
		1:30	(+)	W	W	W	W	W	W	W
		1:40	W	W	W	–	–	–	–	–
H	anti-H	1:80	4+	4+	4+	4+	4+	4+	4+	4+
	(Rabbit)	1:160	+	+	(+)	W	W	W	W	W
MN	anti-M	Undiluted	4+	4+	4+	4+	4+	4+	4+	4+
		1:10	4+	3+	3+	3+	3+	3+	3+	3+
MN	anti-N	Undiluted	4+	4+	4+	4+	4+	4+	4+	4+
		1:10	2+	2+	2+	2+	2+	2+	2+	(+)

DISCUSSION

Lubinski and Portnuff proved by partial hemolysis in hypotonic saline solutions that the loss of D agglutinogen was not due to hemolysis, and they suggested three possible theories to explain their results:

(1) The D antigen may be nearer the cell surface and the A, B, M, and N agglutinogens further within the cell. (2) There may be some chemical differences to account for the different behavior. (3) The Rh agglutinogens may be less numerous than the A, B, M, and N agglutinogens as had been previously suggested by Wiener and associates.[4]

Similar theories are applicable to the C, E, e, Le^a, and Le^b agglutinogens.

Morgan and Watkins[5] have shown that the H antigen is closely related to the ABO groups. It is not surprising, therefore, that the H antigens behave similarly to the A and B antigens. A more unexpected result is the behavior of the c antigen; the antigens E and e are both thermolabile, whereas the comparable antigens C and c show different reactions to heat. It was not possible to test the d antigen's reaction owing to the absence of anti-d sera. The exceptional behavior of c suggests that Lubinski and Portnuff's chemical explanation is the most likely in the Rh groups as it would be unlikely that one allelomorph of a series would produce an antigen less numerous or differently placed within the cell than the remaining allelomorphs of the group.

The effect of heat on the agglutinogens is in no way related to the optimum temperature of their reactions with their antisera as, although A, B, M, N, and H are all active at room temperature, the Lewis agglutinogens (acting optimally at 17 to 18° C.) are not heat stable.

The loss of the power of cells to be agglutinated is gradual, which suggests that the destruction or change is a gradual process. The labile antigens are impaired by heating in a similar manner to the impairment of antiserum by dilution seen in the ordinary titration of the antiserum against unheated cells.

This may mean that (1) the antigen is reduced more or less uniformly throughout the cell suspension, or (2) that the antigen of some individual cells is destroyed sooner than that of other cells.

The dilution experiments provide a means of varying both the strength of antigen and antisera at the same time. This is illustrated in Table II where, using unheated cells and both undiluted and diluted serum, an exactly similar agglutinate is obtained, but by using heated cells the undiluted serum produces a smaller agglutinate while the dilute serum gives a negative result. It is apparent that the size of agglutinate must be related directly to both the strength of antigen and antiserum simultaneously.

Besides theoretical interest the results of this investigation have some bearing on practical blood group serology. The procedure of eluting cells in saline at 56° C. has been applied to the investigation of acquired hemolytic anemias to remove strong autoagglutinins and to remove antibodies from the surface of the cells of infants suffering from haemolytic disease of the newborn; it is then usual to check the Rh groups of the cells. The heating usually recommended is five minutes. The present investigations suggest that this period should not be exceeded if Rh or Lewis typing is to be done (except for antigen c) and even then high titer antisera should be used if reliable results are to be obtained. The results also emphasize the need to avoid heating blood samples for grouping during transit and that nursing and other staff should be instructed not to place samples on or near radiators, etc.

SUMMARY

The effect of heat on the C, D, E, c, e, Le^a, Le^b, A, B, H, M, and N agglutinogens was investigated.

The D antigen was confirmed to be heat labile and the A, B, M, and N antigens confirmed to be heat stable.

The C, E, e, Le^a, and Le^b agglutinogens were found to be heat labile. The c and H agglutinogens were found to be heat stable.

The significance of these results is discussed.

I am indebted to Dr. R. H. Malone, Director of the Regional Blood Transfusion Laboratory, Sheffield, for permission to publish this investigation and for much helpful criticism. I also wish to thank Mr. I. Dunsford, Chief Technician, for making available to me the necessary cells and sera and for his ready advice at all times.

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ELECTROPHORETIC STUDIES ON SERUM AND URINE PROTEINS IN NEPHROSIS TREATED WITH ACTH

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THE demonstration of favorable alterations of serum proteins in nephrotic patients treated with adrenocorticotropin (ACTH, Armour) led to an analysis of sequential changes in the protein fractions. Concomitant analyses of urine proteins were followed out in order to investigate detailed relations in serum and urine proteins. The study was thus directed toward the following inquiries: (a) relations of urine protein fractions to serum protein fractions in the untreated nephrotic syndrome; (b) the effect of ACTH upon the individual serum protein fractions and upon the individual urine protein fractions; (c) correlations of serum fractions identified with lipoprotein bonds, and the serum cholesterol.

METHOD

Serum and urine samples, taken from patients before, during, and after administration of ACTH, were analyzed with the Tiselius electrophoretic apparatus as modified and described by Moore and White.[1] Longsworth's[2] scanning method is employed, using rather than his single knife edge a double knife edge which produces on the photographic plate two patterns whose areas, when averaged, give a value independent of exposure.

The sample (serum, diluted two to four times with buffer depending on its protein concentration previously determined chemically, or urine undiluted) was dialyzed overnight or longer against a sodium veronal buffer, pH 8.6, μ 0.15, as recommended by Longsworth,[3] and run one and one-fourth to two and three-fourth hours, keeping the energy dissipated by passage of current within about one watt so that heat losses could be easily controlled with the 0° to 2° C. water bath. The time and current in each case were noted, and conductances were determined. From these data were calculated relative mobilities (used simply as a means of identification and purposely omitted from the results tabulated subsequently).

Pictures of the ascending and descending patterns were taken at the end of the time interval. The patterns, enlarged at a constant focal length, were traced on drawing paper, and the areas under the peaks were determined with a planimeter.

Ten subjects were studied, of whom seven were 17 years of age or under. All patients were examples of simple nephrosis without marked features of renal damage. Two of the ten were without manifest edema (R. B. and G. H.) (Table I). All patients were maintained on a 700 mg. salt intake.

RESULTS

In Table I are found urine protein values in terms of percentage component, and serum protein values in terms of planimeter units and percentage component. Serum cholesterol values in milligrams per 100 ml. are also included. Mention is made of the disparity between total planimeter units and total pro-

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TABLE

PATIENT	DATE	SERUM-ALBUMIN P.U.	SERUM-ALBUMIN %	α‡ D1 AND D2 P.U.	α‡ D1 AND D2 %	β P.U.	β %	γ P.U.	γ %
Normal	1*		64.0		8.8		14.7		12.4
	2†		66.5		8.8		9.3		15.4
	3†		68.2		9.2		10.7		11.9
D. D.	1- 8	3.54	25.9	6.96	50.8	2.33	17.0	0.86	6.3
	1-24	11.96	64.0	1.74	9.4	2.06	11.0	2.92	15.6
W. D.	5-17	2.56	20.3	3.98	31.5	5.74	45.4	0.36	2.8
	6-13	8.52	65.2	1.96	14.9	1.71	13.1	0.89	6.8
L. T.	7- 5	7.24	46.8	2.84	18.4	3.54	23.0	1.82	11.8
	7-18	8.18	47.1	3.14	18.1	3.95	22.8	2.08	12.0
	8-22	10.84	65.0	2.08	12.5	2.30	13.8	1.46	8.7
	11-27	10.90	64.4	1.70	10.1	2.15	12.7	2.23	13.2
R. F.	5- 9-50	3.16	25.7	0.48 3.9	4.45 36.2	4.04	32.8	0.17	1.4
	6-26-50	1.68	13.0	8.29	64	2.70	20.9	0.27	2.1
	8-28-50	1.84	13.9	0.38 2.8	5.88 42.5	5.50	40.8	0	0
	2-20-51	1.90	15.5	0.58 4.8	2.80 22.8	6.96	56.9	0	0
	3-12-51	3.60	43.6	2.25	27.3	2.40	29.1	0	0
T. K.	1-29-51	4.72	44.8	0.80 4.0	3.86 17.5	3.84	20.2	0.70	13.5
	2-12-51	5.08	35.4	5.52	38.5	1.94	13.5	1.80	12.6
	3- 6-51	5.80	44.8	0.52 4.0	2.26 17.5	2.62	20.2	1.74	13.5
M. M.	5-29	2.43	19.4	0.75 6.71	6.71 52.8	1.56	12.5	1.17	9.3
	6-19	2.70	22.5	0.65 4.67	4.67 38.9	2.97	24.7	1.02	8.5
	6-26	3.42	27.0	0.52 6.72	6.72 53.0	1.68	13.2	0.34	2.7
	12-12-50	5.37	44.5	0.42 3.5	4.4 36.4	1.65	13.7	0.23	1.9
	1-15-51	5.72	43.7	4.59	35.1	2.19	16.7	0.59	4.5
	2-12-51	6.36	51.2	0.36 2.9	3.92 31.5	1.60	12.9	0.18	14.5
G. H.	4-24	5.64	41.8	3.68	37.3	3.26	24.2	0.91	6.7
	6-19	7.56	59.3	2.43	19.0	1.8	14.1	0.97	7.6
R. B.	3-27	5.34	41.9	2.58	20.2	3.90	30.6	0.93	7.3
	5- 1	5.81	37.5	3.36	21.7	4.87	31.4	1.47	9.5
	5-24	4.44	47.8	2.28	24.6	2.34	24.8	0.23	2.8
	6-13	5.81	45.5	3.02	23.7	3.20	25.0	0.73	5.8
A. G.	10-24	2.56	30.3	2.52	21.3	3.46	35.1	1.32	13.4
	12-18	3.44	31.9	2.34	21.7	3.42	31.7	1.58	14.7
D. H.	9- 5	6.16	52.0	2.30	19.3	2.76	23.2	0.66	5.5
	9-18	6.16	55.6	2.00	18.1	2.00	18.1	0.90	8.2
	9-25	6.26	53.2	2.12	18.0	2.24	19.0	1.16	9.8
	11-21	6.84	61.4	1.36	12.2	1.82	16.3	1.12	10.1
L. B.	8-21	5.97	43.2	2.28	16.5	3.80	27.5	1.76	12.8
	9-11	5.90	44.3	2.94	22.0	3.74	28.1	0.74	5.6
	9-18	5.76	39.3	2.92	19.9	3.96	27.0	2.02	13.8
	10- 3	6.66	43.0	3.12	20.1	4.14	26.8	1.56	10.1

*From Longsworth.[4]
†From this laboratory.
‡Where two values are given, α_1 and α_2 peaks were measurable.

I

URINE										
TOTAL PROTEIN		CHOLESTEROL								
P.U.*	GM./100 ML.	MG./100 ML.	DATE	A (%)	α‡ (%)	β (%)	γ (%)	TOTAL PROT. GM./L.	ACTH R DATE/MG./24 HR.
13.69	4.4	625	1-10	71.5	7.1 3.4	4.6	13.4	16.1	1—13-17, 75
18.64	6.5	216	1-16	73.3	11.3 2.5			15.5	
			1-20	100				1.8	
12.64	3.9	745	5- 9	70		17.8	12.2	2.1	5—11-22, 50
13.08	6.6	218	5-15	100				0.8	5—23-30, 25
			6-18	95		5.0		2.9	6—24 to 7—6, 50
			6-28	0				0	
15.44	4.8	531	7- 4	100				0.86	7—8-19, 50
17.35	5.0	477	7-12	100				2.70	7—20, 21, 25
16.68	6.4	197	7-28	0				0	
16.98	7.0	265							
12.30	5.4	785	4- 4-50	54.7	25.0	10.5	9.8	14.4	6—13-20, 50
12.94	3.0	1045	4- 7	48.0	26.3	7.1	18.6	13.8	6—21, 22, 75
13.68	4.0	1005	4-24	34.1	42.2	8.5	15.1	13.5	7—3-13, 75
12.04	3.7	860	5- 6	79.0	10.0	11.0	0	2.7	8—9-18, 75
			6-12	78.2	6.0	6.4	9.4	4.1	
8.25	5.1	525	2- 9-51	67.0	11.0 5.9	6.6	9.5	9.8	
			3-14	100				1.8	
13.92	4.4	550	1-10-51	67	3.1 7.9	10.6	11.5	10.69	1—8-15, 50
14.34	4.7	438	2-24	68.7	31.8			1.1	1—22 thru 2-8, 50
12.94	5.4	345	3- 6	100				2.9	2—14 thru 3-5, 50
12.62	3.9	731	5-15	65	10.0	9.5	15.5	4.0	5—29—6-6, 50
12.01	3.4	784	5-20	71.7	9.7	7.2	11.4	11.5	6—10-15, 50
12.68	4.8	771	5-25	100				2.6	6—16, 100
12.07	5.1	612	6-26					3.4	
13.09	5.5	475	12-12	79.2	8.3	8.3	4.1	4.2	
12.42	5.9	413	2-23	76.9	16.0	7.1		1.8	
13.49	4.5	560	4-24	100				2.2	4—28-30, 70
12.76	5.4	333	5-15	97.3	2.7			4.0	5—1-4, 100
			5-21	100				1.1	5—5, 50
			6-19					2.6	5—11-16, 100
12.75	5.2	610	3-27	100				0.9	3—29, 35
15.51	5.9	531	4- 3	100				1.7	3—30—4-1, 75
9.29	4.7	470	4- 9	67.2	32.8			2.4	4—2-5, 100
12.76	5.6	458	5-24					3.5	5—26—6-2, 100
			6-13					4.6	6—3, 4, 50
									6—5, 6, 25
9.86	4.3	756	10- 3	100				4.3	10—5-11, 100
10.78	3.6	500	10-10	69	9.8	9.0	12.2	5.4	10—16-25, 100
			12-22	88.2	5.9	5.9		5.8	
11.88	5.7	380	9- 3	100				2.3	9—6-12, 100
11.06	5.6	215	9- 8	100				2.8	9—13-18, 75
11.70	5.6	119	9-25	100				1.4	10—26, 27, 100
11.14	6.5	204							11—4-7, 50
13.81	6.3	408	8-23	100				2.8	8—23-31, 100
13.32	6.0	440	8-30	100				2.3	9—6-14, 100
14.66	5.6	450	9-15	100				1.3	9—15, 50
15.48	6.4	412							

tein concentration (in grams per 100 ml.) occurring in sera of high cholesterol content. The contribution to the α- and β-globulin fractions made by the lipoid material obviously presents a "false total" in planimeter units. Lack of large quantities of material made extraction, and therefore elimination of this incongruity, impossible.

Throughout the series the control determinations conformed well with reported values.[4-8] These consist of low values for albumin and γ-globulin with markedly elevated values for α- and β-globulin. In those patients (W. D., L. T., D. D.) who underwent complete remissions with ACTH, the serum protein defects were reversed. These advanced without interruption over the period of observation to completely normal values, the γ-globulin in the first two constituting the only exception (Table I).

In those patients in whom remissions were partial or who were inert to treatment with ACTH, the changes in serum protein fractions were variable and inconsistent.

Although the albumin was the principal urine protein, several patients, particularly those with great protein losses, showed complex urine protein patterns. The ratio of components in these examples (D. D., W. D., 5/9, R. F., M. M., A. G.) was approximately the same as that existing in normal serum. The classical teaching that the disturbance in serum protein is the result of urinary protein loss is belied by the considerable urinary globulin in those with markedly elevated serum globulin. Lack of quantitative relationship between serum protein and urine protein is evident in W. D., G. H., R. B., and M. M., where the total serum protein increases substantially in the absence of corresponding amelioration of proteinuria.

Throughout the series, a reduction in α- and β-globulin associated with treatment by ACTH tended to parallel the reduction in serum cholesterol. In the patients who were inert to treatment the α- and β-globulins, and likewise cholesterol concentration, remained essentially the same.

The influence of the adrenal cortex upon protein metabolism has been recognized chiefly in the increased excretion of nitrogen which becomes apparent after three to four days of ACTH administration.[9-12] In the nephrotic series, as in normal subjects, this increase in urinary nitrogen was associated with negative balance, although the effect was often of brief duration (Fig. 1). The negative phase was followed by an extended period of positive balance during which the patient retained 4 to 6 Gm. of nitrogen daily for several weeks. Estimations of urine urea performed in two patients with nephrosis who were receiving ACTH show an increase which coincides with the increase in total nitrogen.

The intensity of this series of reactions has been seen to vary rather widely, as is shown in Fig. 1. Of these two male patients, 13 and 49 years of age, respectively, D. H. experienced a period of marked negative nitrogen balance with an increase in urine urea from a control level of 6.6 Gm. per day to 12.7 Gm. on the ninth day of treatment. R. B., on the other hand, had only a day or two of negative balance, and his urea output increased but slightly.

DISCUSSION

These events fall within the results of ACTH administration which have already received ample demonstration in other disease entities and in normal subjects. In the nephrotic syndrome, however, the collapse of homeostatic mechanisms for the maintenance of normal plasma proteins provides a singular opportunity to observe the effects of cortical stimulation upon protein metabolism.

The balance data with the rising urine urea values suggest that protein metabolism is active following activation of the cortex. The period of plasma protein building which follows in a majority of cases has been seen in routine chemical determinations to proceed regardless of intake and of protein excreted in the urine. Further analysis of these changes by the methods of electrophoresis reveals no characteristic pattern sequence which could be

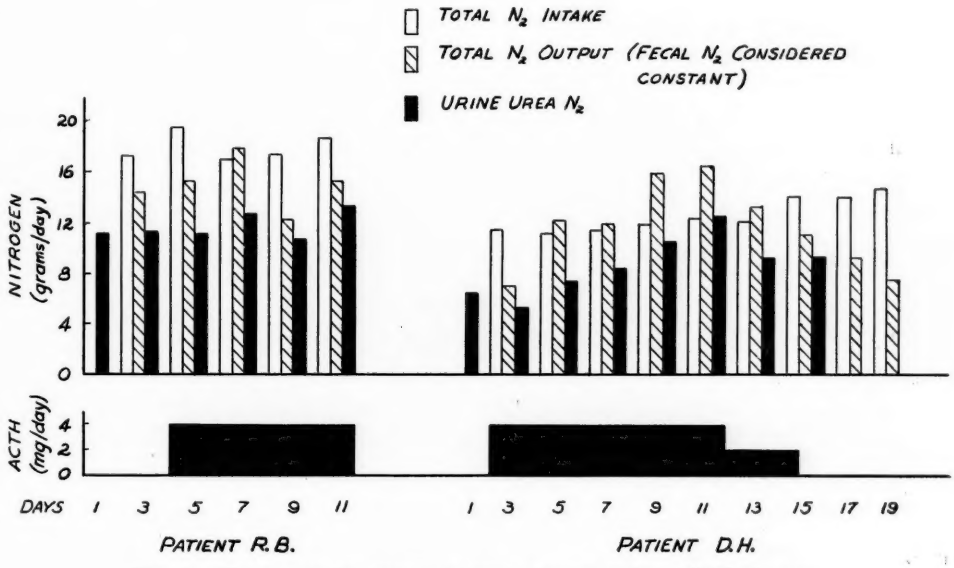

Fig. 1.—Changes in N_2 values in two patients under ACTH therapy.

taken to indicate synthesis of an increment or group of increments, or the inhibition of wasting of increments, possibly by diffusion.[8] In only one of the cases studied did the γ-globulin fraction return to normal, a finding which may be related to an observation made by Eisen and his group: adrenalectomized rats with γ-globulin values 19 to 23 per cent lower than normal were maintained on desoxycorticosterone and adrenocortical extract; they showed no difference in the γ-globulin fraction or in the circulating antibodies.[13]

There is, it is true, a general correlation between decreased cholesterol and decreased α- and β-globulin, presumably through a decrease in the lipoproteins which constitute the fat transport system. The existence of lipids of normal human serum in close association with certain of the serum protein fractions has been observed. Blix, Tiselius, and Svensson[14, 15] studied the

lipid content of electrophoretically separated fractions of human serum and found it associated chiefly with the α- and β-globulins. It is suggested that the β-globulin fraction, which seems to have the highest affinity for most lipids, has a particular function in the fat transport of the body. In marked lipemic conditions, a turbidity appearing between the α- and β-globulin boundaries probably indicates that when larger amounts of fat have to be transported, the α-globulin and possibly albumin go into the surface film of the lipid particles and thus take part in the fat transport. While there is no evidence of albumin or γ-globulin fractions associating appreciably with particulate lipids in plasma,[16, 17] 35 per cent and 75 per cent lipid are assigned to the α- and β-globulin fractions, respectively.[18] The accuracy of the relationship between cholesterol and α- and β-globulin in our data is no doubt disturbed, however, by the fatty acids and other lipids which compete for the transport system, and have been shown to reflect more sensitively changes in blood fats elicited by ACTH.

Thus, we find no indication in electrophoretic analysis of blood and urine proteins of primary effects upon protein synthesis or distribution induced by ACTH, by which the favorable results seen in the nephrotic syndrome could be accounted for. Contrary to effects upon lipids described in the foregoing paper, the improvement in serum proteins seen in successfully treated cases appears to be a delayed and indirect reflection of tissue changes for which more immediate and specific indices are wanting.

CONCLUSIONS

Partition of proteins before, during, and after administration of ACTH indicates that:

1. In the cases successfully treated with ACTH, the serum protein defects were reversed; in those who were inert and in those with partial remissions, serum protein changes were variable.

2. ACTH treatment had no appreciable effect on the γ-globulin fraction; in only one of the cases studied did it rebuild completely.

3. Reduction of the α- and β-globulins of the serum was accompanied in general by a reduction in serum cholesterol.

4. No changes, qualitative or quantitative, in the urine proteins could account for changes in the serum protein patterns.

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SERUM LIPID ANALYSIS IN THE NEPHROTIC SYNDROME UNDER ACTH ADMINISTRATION

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WE HAVE found a marked decrease in serum cholesterol in patients with the nephrotic syndrome under treatment with adrenocorticotropin (ACTH, Armour).[1, 2] Reports on the effect of ACTH in normal individuals with respect to serum lipids have indicated variable and, on the whole, moderate changes.[3-5] The present analysis of lipid fractions was therefore set up with the following purposes: (1) to obtain data relative to the sources of hyperlipemia in the nephrotic syndrome, and (2) to employ the nephrotic hyperlipemia to obtain information on the effect of ACTH on fat metabolism.

METHODS

In general, the method of differential lipid assay was that employed by Popjak.[6] The sera were extracted by the technique of Bloor;[7] the phospholipids present in the redissolved residue were precipitated with acetone and magnesium chloride and analyzed by a modification of the method of Fiske and Subbarow. In the supernatant, total and free cholesterol are determined by a modification of the Schoenheimer-Sperry procedure, as proposed by Clarke and Marney.[8] The same phospholipid-free supernatant is used for triglyceride and cholesterol ester assay after conversion to the chromogenic hydroxamic acid derivatives of the fatty acids.[9] Thus, free and total cholesterol, phospholipids, and nonphospholipid fatty acids are determined directly. Total serum lipids, cholesterol ester fatty acids, and triglycerides are calculated from these data. Duplicate determinations of lipid phosphorus were run on separate 10 ml. aliquots of the serum extract. The phospholipid-free supernatants of these were then combined and analyzed against an identically processed 20 ml. aliquot of the original extract as a duplicate for the cholesterol and esterified fatty acid determinations. Duplicates of all agreed well in the various ranges of serum lipids dealt with. Where values showed precipitate drops, analysis on subsequent unreported sera were run, and demonstrated close proximity. Quantitative recoveries were obtained with this method on cholesterol and esterified fatty acids.

This method of aggregate colorimetric analysis favors the admission of any error into the systematic class, an argument originally proposed by Boyd in relation to his aggregate dichromate oxidation method.[10]

CLINICAL MATERIAL AND RESULTS

Six patients ranging in age from 3 years to 58 years, who typify the nephrotic syndrome, constituted the series. All subjects were placed on a diet containing 700 mg. of sodium.

Case 1.—T. K., 3-year-old boy (Table I), whose disease was of two years' duration, showed the most profound changes in serum lipids. Control determinations showed, in milligrams per cent: free cholesterol 421, total cholesterol 1,291, cholesterol ester fatty acids 626, phospholipids as lecithin 702, non-

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TABLE I. T. K., BOY, 3 YEARS*†

DATE	CHOLESTEROL			PHOSPHOLIPID (AS LECITHIN)	NONPHOSPHO-LIPID EFA	TRIGLYC-ERIDES	TOTAL LIPID
	FREE	TOTAL	ESTER FA				
Control	421	1,291	626	702	4,920	4,510	7,129
Nov. 6	278	956	488	660	3,960	3,640	5,744
Nov. 13	230	832	433	648	2,250	1,910	3,823
Nov. 20	193	804	440	548	473	35	1,827
Nov. 27	196	740	392	676	853	484	2,292

*ACTH—50 mg. per day Oct. 31 to Nov. 4; 50 mg. per day Nov. 10 to 22; 25 mg. per day Nov. 23 to 26.

†All values quoted in the tables are in milligrams per 100 ml.

phospholipid fatty acids 4,920, triglycerides 4,510, and total serum lipids 7,129. In three weeks the triglycerides dropped to 34.7 mg. per cent. Phospholipids and cholesterol decreased concomitantly but to a far lesser degree. The marked improvement in serum lipids was accompanied by a complete diuresis, with a rise in serum proteins and clinical evidences of well-being. The result was classified as a partial remission.

CASE 2.—M. L. M., 10-year-old girl. (Table II), who had been well until 1944, showed a similar course in respect to the general trend of lipids, although cholesterol and lecithin showed less effect. As in the preceding case, triglyc-

TABLE II. M. L. M., GIRL, 10 YEARS*

DATE	CHOLESTEROL			PHOSPHOLIPID (AS LECITHIN)	NONPHOSPHO-LIPID EFA	TRIGLYC-ERIDES	TOTAL LIPID
	FREE	TOTAL	ESTER FA				
Sept. 19	235	902	480	462	1,225	782	2,626
Sept. 25	214	770	401	467	1,100	734	2,372
Oct. 2	197	822	450	395	947	522	2,189

*ACTH—75 mg. per day Sept. 21 to 29.

erides and total lipids declined markedly. In this case, as in T. K., diuresis occurred during the experimental period; unlike the first case, however, no significant change was found in the serum proteins. Proteinuria continued undiminished.

CASE 3.—D. H., 13-year-old boy, was taken ill in December, 1949. The lipid disturbance as reflected by serum findings was of lesser degree, the first control specimen having showed a cholesterol of 435 mg. per cent. Table III shows data collected from the second course of treatment. The control values at this time reflected approximately normal values, the triglyceride figure of 187 being the only component significantly above normal quotations. The first determination of triglycerides following commencement of the ACTH regime showed a dramatic drop, reminiscent of T. K., but this was not maintained on a decreased dose. There was more actual fluctuation in the individual lipid fractions than the total lipids, which totals fell within 5 per cent of the mean of all. Notwithstanding the less conspicuous lipid disturbance and the transient result from ACTH therapy, the clinical course of this subject was characterized by complete diuresis, with a moderate decrease in proteinuria and a slight increase in serum proteins.

CASES 4, 5, AND 6.—A. G., R. B., and L. B., ages 42 years, 48 years, and 58 years, respectively, have been grouped together because of the common fac-

tors of duration of illness and age group. (Tables IV, V, VI.) A. G. showed insignificant changes in cholesterol fractions, but an eventual steady reduction in triglycerides while on therapy. This was correlated with failure of diuresis and unaltered proteinuria and hypoproteinemia. R. B. showed a decline in triglycerides following therapy, a finding which was correlated with a substantial rise in serum proteins. This patient had no evidences of edema upon admission, and changes in body weight were negligible throughout the course. L. B. demonstrated a brief decline in triglycerides, with a final rise in all components. The clinical course was correspondingly uneventful. These three subjects were chosen, not as representative of nephrosis patients in general, but of the age group over 40 years.

TABLE III. D. H., BOY, 13 YEARS*

DATE	CHOLESTEROL			PHOSPHOLIPID (AS LECITHIN)	NONPHOSPHOLIPID EFA	TRIGLYCERIDES	TOTAL LIPID
	FREE	TOTAL	ESTER FA				
Control	96	215	87	222	264	187	710
Oct. 30	65	302	171	218	228	60	751
Nov. 6	60	225	133	218	250	123	699
Nov. 13	57	261	147	228	264	123	759

*ACTH—100 mg. per day Oct. 27 to Nov. 3; 50 mg. per day Nov. 4 to 7.

TABLE IV. A. G., WOMAN, 42 YEARS*

DATE	CHOLESTEROL			PHOSPHOLIPID (AS LECITHIN)	NONPHOSPHOLIPID EFA	TRIGLYCERIDES	TOTAL LIPID
	FREE	TOTAL	ESTER FA				
Oct. 4	188	756	419	354	676	270	1,799
Oct. 10	252	760	366	482	652	300	1,908
Oct. 17	208	752	392	394	602	221	1,759
Oct. 24	190	772	419	383	511	97	1,671
Oct. 30	147	530	276	338	297	22	1,166

*ACTH—100 mg. per day Oct. 6 to 12 and Oct. 17 to 26.

TABLE V. R. B., MAN, 48 YEARS*

DATE	CHOLESTEROL			PHOSPHOLIPID (AS LECITHIN)	NONPHOSPHOLIPID EFA	TRIGLYCERIDES	TOTAL LIPID
	FREE	TOTAL	ESTER FA				
Oct. 12	145	648	382	404	588	216	1,650
Oct. 17	113	520	293	331	500	218	1,362
Oct. 24	117	546	309	381	588	293	1,529
Nov. 7	106	562	329	380	506	186	1,457

*ACTH—100 mg. per day Oct. 13 to 22.

TABLE VI. L. B., WOMAN, 58 YEARS*

DATE	CHOLESTEROL			PHOSPHOLIPID (AS LECITHIN)	NONPHOSPHOLIPID EFA	TRIGLYCERIDES	TOTAL LIPID
	FREE	TOTAL	ESTER FA				
Aug. 22	113	408	212	312	671	482	1,414
Sept. 5	105	440	242	350	688	469	1,501
Sept. 15	132	450	229	289	737	534	1,502
Nov. 13	107	412	220	371	792	600	1,603

*ACTH—100 mg. per day Aug. 23 to 31; 100 mg. per day Sept. 6 to 15.

In summary, three of six patients showed a significant decrease in serum cholesterol, roughly paralleled by the phospholipids. The fatty acid esters of

cholesterol and the nonphospholipid fatty acids concurrently decreased. The serum triglycerides, on the other hand, and with them the total lipids, showed a drop in five of six patients.

DISCUSSION

It will be noted that our control findings agree with those of other investigators. Hiller and his group[11] found lipid elevations in all fractions before test fat meals.

Although the majority of our patients have been shown to demonstrate a notable drop in serum cholesterol under treatment with ACTH, we have included in this group three subjects in whom a decrease did not appear. It is thus evident that triglyceride changes were more closely associated than cholesterol with ACTH-induced cortical activity. The interpretation of this finding depends upon the distribution and functions of lipid components in normal and lipemic serum. A review and discussion of current opinion on this rather complex subject by Frazer[12] furnishes a background summary of fat transport and metabolism of which we have made use as reference points in the evaluation of data here presented.

According to this author, lipids are present in normal human blood plasma in three main forms; fatty acids and their glyceryl esters, phosphatides, and sterols. All three classes are capable of association with suitable proteins as lipoprotein, distinguishable as particulate, and dissolved or molecular. The physiologic lipemia of the postabsorptive state is characterized by large amounts of long-chain triglycerides which are formed in the intestinal mucosa and pass via the lymph into the venous blood at the level of the heart from which it is carried in the systemic circulation to fat depots. There is also good evidence that it is chiefly in the form of triglycerides that fat is conveyed from the depots to the liver and from the liver to the body cells for combustion. The serum concentration of fatty acids as triglycerides, then, can be taken to refer, in general, to the transport system; and the character of the lipo-protein by which the triglyceride particles are stabilized appears to determine the destiny of the particle whether for oxidation, structural function, or passage through membranes.

On the basis of these concepts, it will be readily seen that values for triglycerides in the fasting nephrosis patient ranging from 187 mg. per 100 ml. (D. H., Table III) to 4,510 mg. per 100 ml. (T. K., Table I) compare strikingly with the range of 50 to 100 mg. per 100 ml. characteristic of the normal fasting subject.

The nephrotic lipid pattern might result from five possible mechanisms: (1) increased absorption, (2) increased synthesis from carbohydrate or protein, (3) decrease in oxidation, (4) increase in mobilization, or (5) decrease in deposition.

The first possibility can be discarded because of the normal nearly complete absorption of fats from the gut. Even the postabsorptive hyperlipemia subsides within seven hours.[13]

Increased synthesis of fat from carbohydrate is hardly compatible with the normal values consistently obtained for blood sugar in this series of patients. These findings are in agreement with earlier reports in which the renal threshold for glucose was found to be somewhat lowered when the sugar was administered by the oral route, but the blood level was normal. Likewise, the considerable volume of observation and experiment on the relation of proteinuria or hypoproteinemia to lipemia has not resulted in the establishment of a cause-and-effect relationship, either direct or indirect.

A decrease in fat disposal by oxidation has been ruled out by Hiller, Linder, Lundsgaard, and Van Slyke,[11] who showed an R. Q. depression in fat-fed nephrotics of the same order as in normals.

For these reasons it seems more plausible to consider (1) a decrease in deposition, or (2) an increase in mobilization as a source of the lipids which we find pooled in the transport system. Studies reported by Popjak[6] in 1946 showed that the feeding of amorphous cholesterol in aqueous suspension resulted in an increase in all blood lipid fractions and in a depletion of fat reserves, in the rabbit. The mechanism by which the serum concentration of cholesterol could influence mobilization of fat from depots was unknown. Assuming that the situation in man is analagous to that existing in the rabbit, an increase in fat mobilization would carry the weight of indirect evidence as against a decrease in deposition. Again, accepting as a working hypothesis that the hyperlipemia of the nephrotic syndrome derives largely from fat mobilized from depot stores, the reduction of hyperlipemia by activation of the adrenal cortex suggests that the cortical steroids constitute a factor in the scheme proposed by Popjak.

The fact that Popjak's studies suggest that cholesterol is the key substance to which the neutral fat and the phospholipids respond in turn, whereas our data indicate that changes in mobilization occur more readily than changes in cholesterol concentration, appears as an inconsistency which should receive further study.

Experiments on the adrenal cortex in relation to fat metabolism are too fragmentary to supply a background physiology to which these findings can be readily related. Certain reports, however, may prove to be relevant. Verzar and Laszt[14] were unable to produce fatty livers in adrenalectomized animals by various experimental methods. Goldzieher[15] concluded that adrenal extract causes fixation of blood fat in tissues. Kendall has reported increased carcass fat in rats treated with three pure adrenal steroids.[16] Hyperplasia of the cortex, as well as Cushing's syndrome, is a precursor of obesity of a particular type. It would be compatible with these reports that a decrease in triglycerides should follow the activation of the adrenal cortex by ACTH.

CONCLUSIONS

Partition of lipids in nephrosis during and after administration of ACTH indicates that the triglyceride fraction decreases substantially under activation of the adrenal cortex.

The triglyceride fraction appears to be the most sensitive of the lipid fractions to stimulation of the adrenal cortex.

Since the triglycerides are primarily concerned with the fat transport system, this finding is interpreted as indicating a greatly enlarged transport pool in the untreated nephrotic state. Indirect evidence is proposed which attributes this condition to increased mobilization from fat depots. The response of the triglycerides to ACTH administration is taken as experimental evidence that the cortical steroids are concerned with fat mobilization.

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OBSERVATIONS ON ABSORPTION, DISTRIBUTION, AND EXCRETION OF ELKOSIN (N′-[2,6-DIMETHYL-4-PYRIMIDYL]-SULFANILAMIDE) IN MAN

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WITH the development of efficacious, relatively nontoxic antibiotics, the sulfonamides have been relegated to a position of secondary importance. Despite the known disadvantages of the sulfonamide compounds, they still may occupy a definite place in the physician's armamentarium, singly or in combination with various antibiotics or other sulfonamides.

Ideally, a chemotherapeutic agent should be readily absorbed after oral administration, not appreciably inactivated in the body, and excreted gradually with a minimum of undesirable side effects. Recent investigations have been particularly concerned with improving the solubility of these compounds in the urinary tract. Preliminary studies[1-4] with "Elkosin,"* (N′-[2,6-dimethyl-4-pyrimidyl]-sulfanilamide—2, 4 dimethyl-pyrimidine), have indicated that this drug is exceptionally soluble and therefore less likely to cause renal damage. Since the judicious use of any such agent requires an understanding of its action in man, the following preliminary studies in relation to absorption, distribution, and excretion were conducted.

METHODS AND MATERIALS

"Elkosin" levels in blood, urine, and other body fluids were determined by the method of Bratton and Marshall[5] except that water instead of saponin was used to lake the blood. The values were determined colorimetrically in a Lietz photelometer and calculated from the curve established with a standard "Elkosin" solution. The fate of a single oral or intravenous dose calculated to be approximately 0.05 Gm. per kilogram was studied in twenty individuals who were either normal volunteers or had minor illnesses. None had renal, hepatic, or gastrointestinal disease. No attempt was made to regulate the fluid intake or to control the volume or pH of the urine. Base-line blood and urine specimens were obtained before the administration of "Elkosin" and then again at two-, four-, six-, eight-, ten-, twelve-, twenty-four, and forty-eight hour intervals. In addition, spinal fluid was taken after four and/or eight hours whenever possible.

RESULTS

1. *Oral Administration.*—The blood levels observed at each time interval in ten persons receiving a single oral dose of "Elkosin" are illustrated graphically in Fig. 1. The highest blood concentrations were noted after two, four and eight hours in five, four, and one patients, respectively; the maximum total "Elkosin" levels ranged from 7.3 to 11.8 mg. per cent, with an average peak value of 10.3. At these times, 91.2 to 98.3 per cent (average 95.3 per cent) of the drug was present as free "Elkosin." After twelve hours, total values

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*Ciba's trade-name for N′-(2,6-dimethyl-4-pyrimidyl)-sulfanilamide.

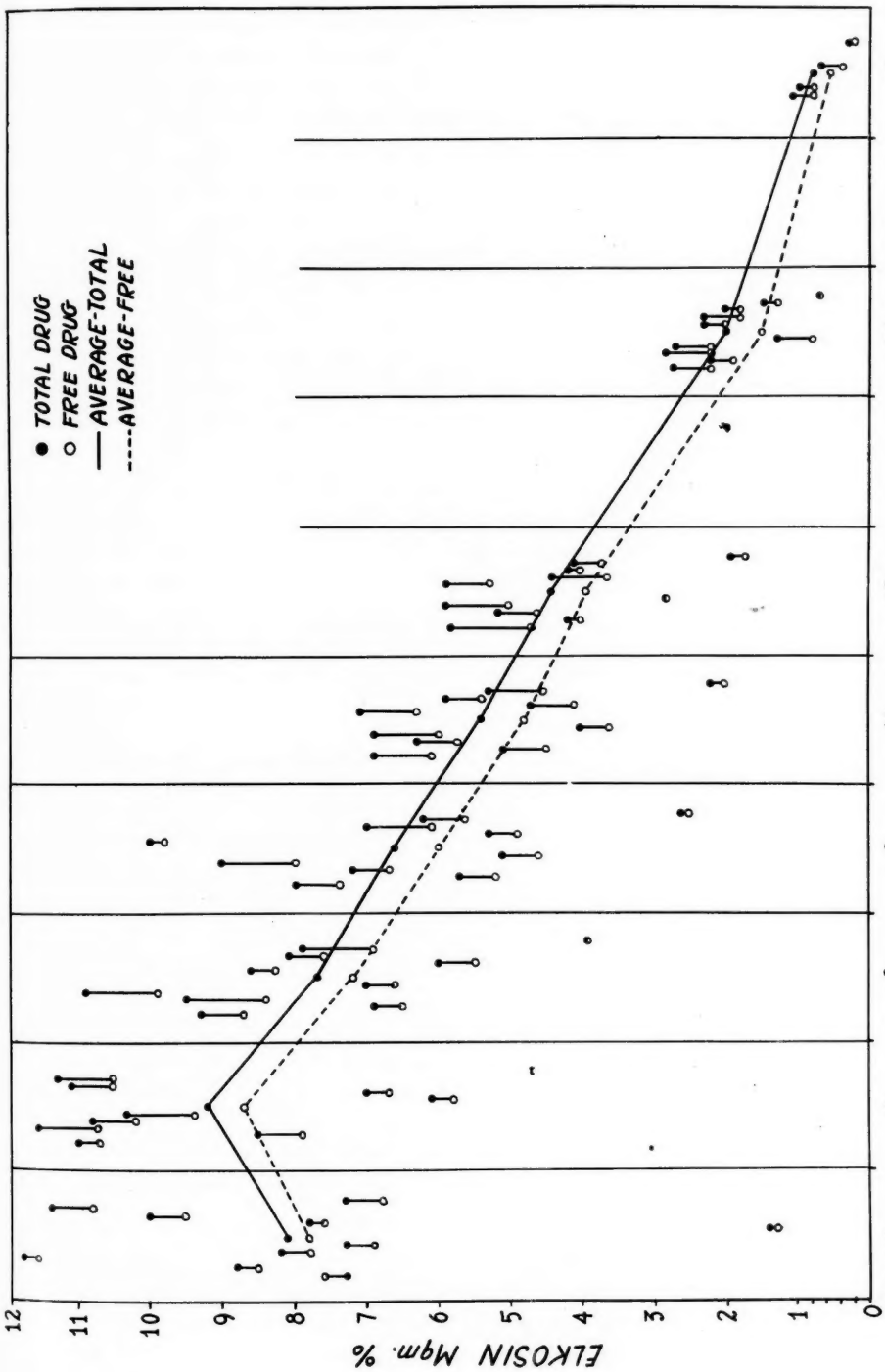

Fig. 1.—Elkosin blood levels following single oral dose of 0.05 Gm. per kilogram.

varied from 1.9 to 5.9 mg. per cent (average 4.4 per cent) and an average of 90.1 per cent free drug was observed at this time with a range of 84.8 to 98.2 per cent. After twenty-four hours, total "Elkosin" varied from 0.7 to 2.8 mg. per cent (average 2.1 mg. per cent); 61.4 to 100 per cent was non-acetylated with an average of 83.1 per cent. In four cases where forty-eight hour specimens were obtained, an average total level of 0.79 mg. per cent with 73 per cent free "Elkosin" was observed. The results as depicted in Fig. 1 demonstrate the comparative values of free and total blood "Elkosin" observed in seventy-four separate specimens over a forty-eight hour period. The free "Elkosin" values ranged from a low of 55.5 per cent (forty-eight hour specimen) to 100 per cent, with an average of 90 per cent.

The maximum urinary excretion of the drug occurred after two, four, six, and eight hours in three, four, two, and one patients, respectively. At these times the average peak total urinary concentration was 306 mg. per cent, of which 92.3 to 100 per cent (average 96.8 per cent) was in the free form. After twenty-four hours 70 to 98.8 per cent (average 86 per cent) of the total ingested drug had been excreted in the urine. Over a twenty-four hour period an average of 89.2 per cent was free, with individual specimens varying from a high of 97.5 per cent to a low of 75.8 per cent. After forty-eight hours 90.3 per cent of the drug had been excreted. Specimens saved over the second twenty-four hour period showed 56.7 per cent to be in the free form. However, including the latter results in the grand total of seventy-five separate determinations done over a forty-eight hour period, an average of 87 per cent was present as free "Elkosin." Fig. 2 depicts graphically the comparative free and total urine sulfonamide levels.

Lumbar punctures were obtained from six persons at both four and eight hours after ingestion of the drug, while in a seventh individual a single four-hour specimen was taken. Free "Elkosin" levels of 0.45 to 1.0 mg. per cent were detected at four hours (average 0.76 mg. per cent); these spinal fluid concentrations were 9.95 per cent of the average blood level. At eight hours after a single oral dose the average spinal fluid concentration was 1.2 mg. per cent, with values ranging between 0.85 to 1.5 mg. per cent; thus 22.6 per cent of the concomitant average blood level was detectable in spinal fluid at this time. Although these spinal fluid concentrations may appear to be relatively low, it is obvious that the results were obtained after a single dose in individuals with no meningeal disease. However, in sixteen comparative determinations of blood and spinal fluid in seven patients under continuous "Elkosin" therapy for meningitis, an average of 49.6 per cent of the free blood level was observed in spinal fluid (Table I).

Concentrations of free "Elkosin" in thoracentesis fluid were determined in three patients with pleural effusion. In all three individuals the concentration of free "Elkosin" exceeded that present in the blood. With blood levels of 7.8, 8.0 and 9.2 mg. per cent, effusion concentrations of 9.4, 8.6, and 17.4 mg. per cent, respectively, were observed. More studies are in progress to determine the repeatability and significance of these results.

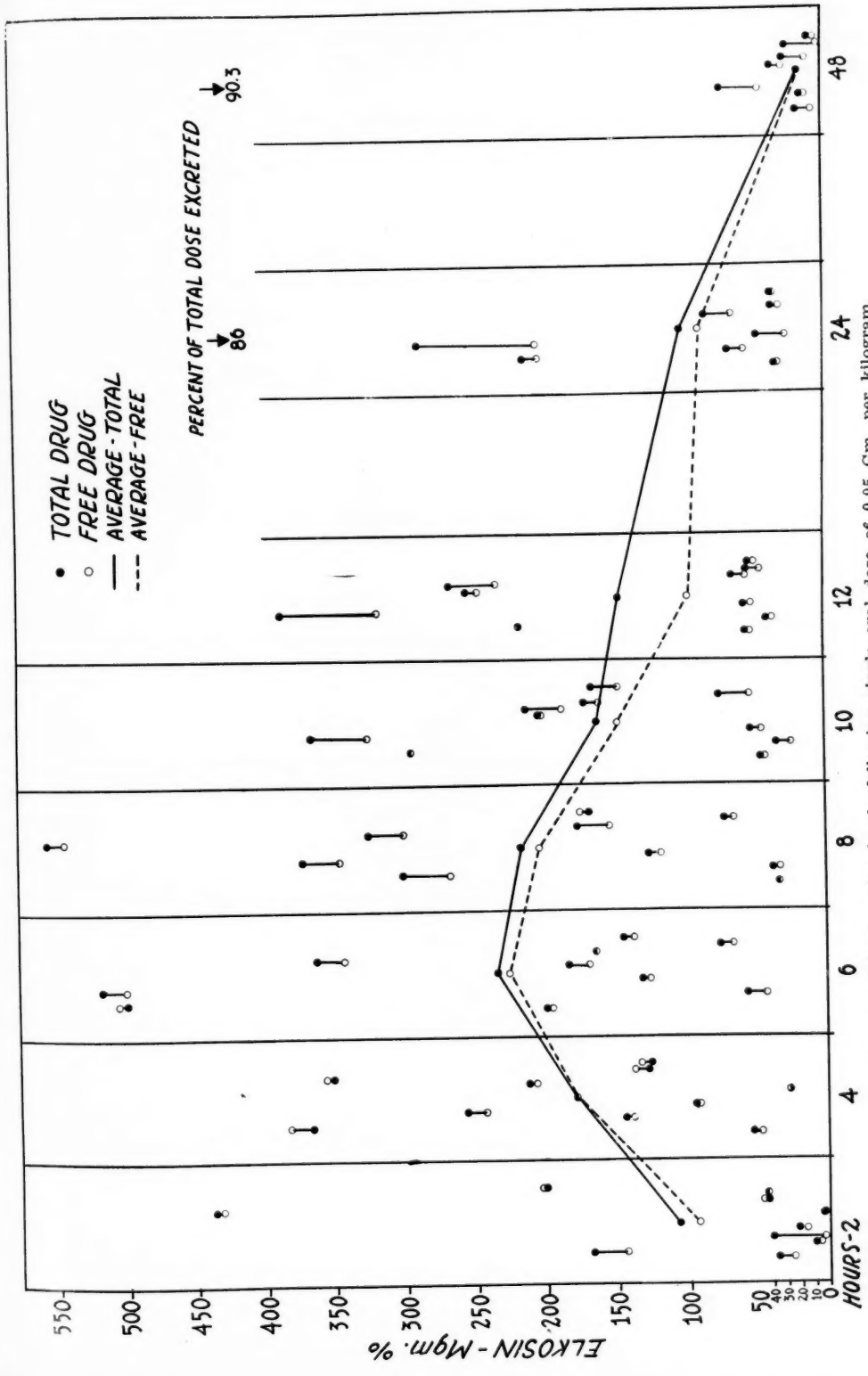

Fig. 2.—Elkosin urine levels following single oral dose of 0.05 Gm. per kilogram.

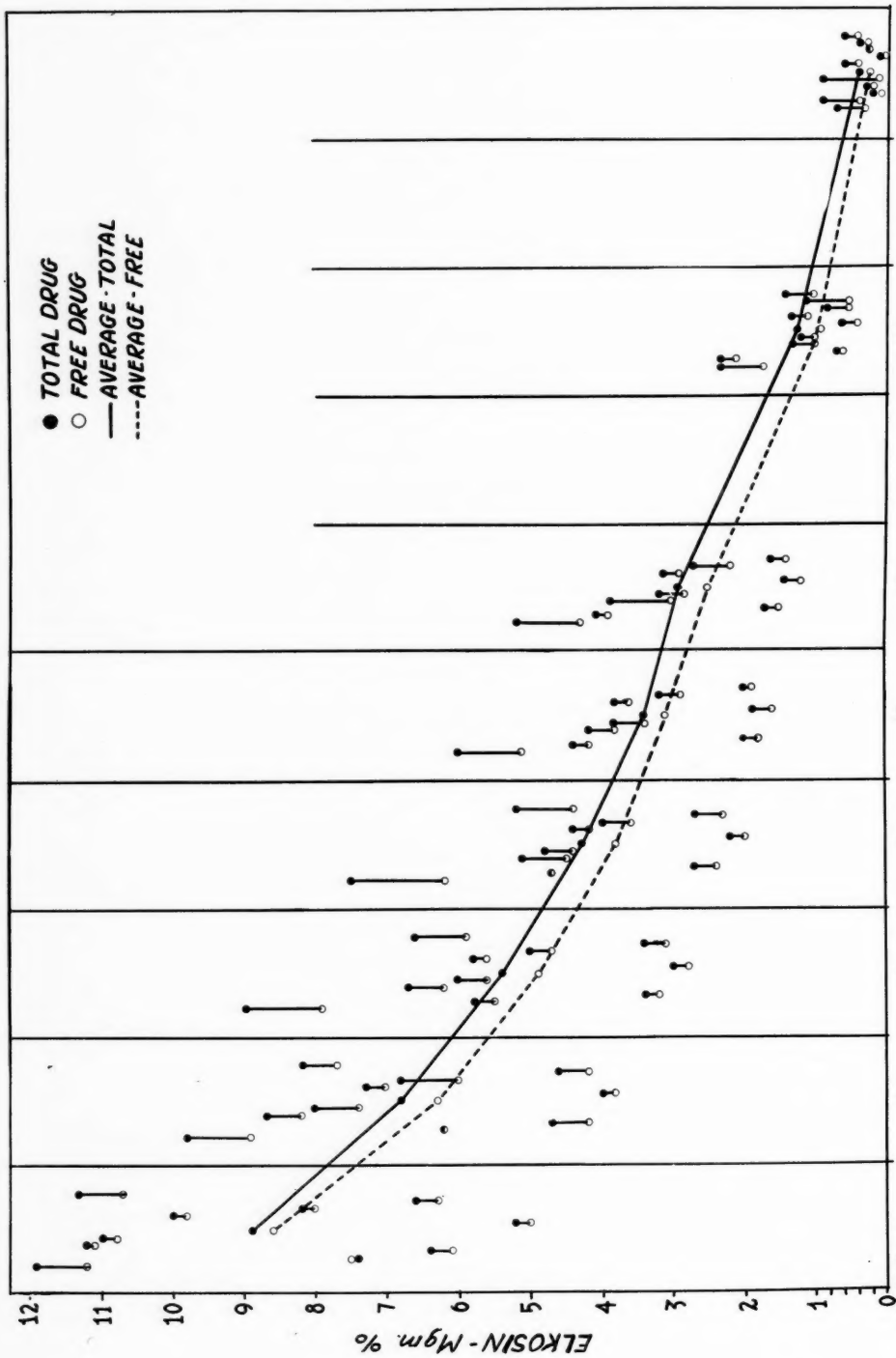

Fig. 3.—Elkosin blood levels following single intravenous dose of 0.05 Gm. per kilogram.

Fig. 3.—Elkosin blood levels following single intravenous dose of 0.05 Gm. per kilogram.

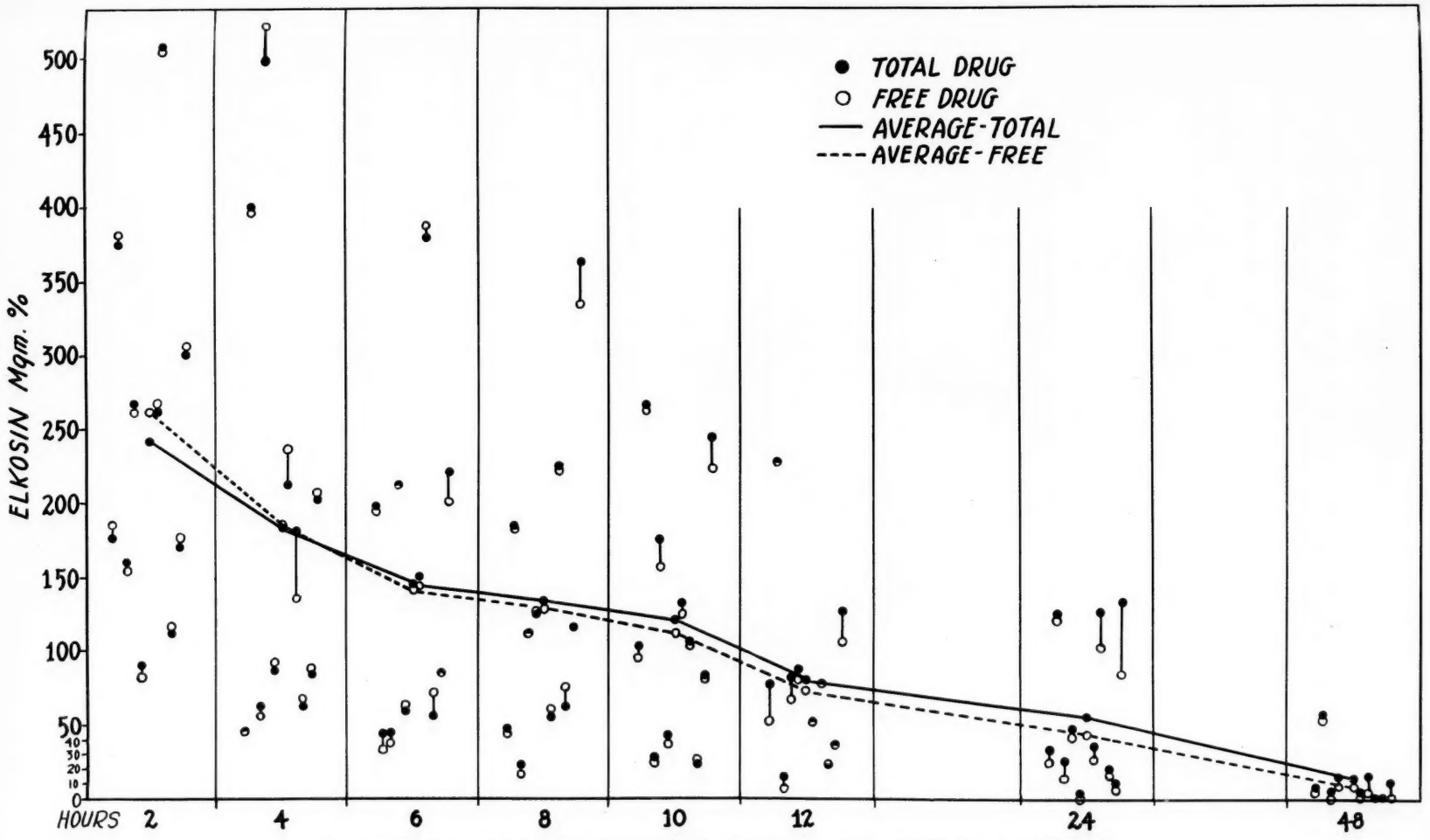

Fig. 4.—Elkosin urine levels following single intravenous dose of 0.05 Gm. per kilogram.

TABLE I. FREE BLOOD AND SPINAL FLUID CONCENTRATIONS OF ELKOSIN FOLLOWING CONTINUOUS ORAL THERAPY IN SEVEN PATIENTS WITH MENINGITIS

PATIENT	SPECIMEN NO.	CONCENTRATION OF ELKOSIN (MG. PER 100 ML.)		
		BLOOD	SPINAL FLUID	% OF BLOOD LEVEL IN SPINAL FLUID
E. C.	1	5.2	1.3	26.0
	2	4.9	2.1	42.9
	3	5.3	2.2	41.5
	4	6.8	2.4	35.2
E. F.	1	19.2	8.0	41.0
	2	20.0	9.5	47.5
	3	19.8	10.6	53.5
	4	31.4	22.8	72.6
	5	29.0	19.2	66.2
M. L.	1	4.3	1.8	41.3
	2	4.8	1.2	25.0
W. B.	1	7.2	2.4	33.3
	2	6.9	1.5	21.7
R. H.	1	10.2	2.2	21.5
D. C.	1	6.6	1.4	21.2
R. E.	1	21.6	12.0	55.5
Average	(16 specimens)	12.7	6.3	49.6

Twenty-four hours after a single oral dose, two patients with ascites exhibited concentrations of free "Elkosin" equivalent to one-third that noted in blood.

2. *Intravenous Administration.*—The blood levels observed at varying time intervals in ten persons receiving a single intravenous dose of "Elkosin" are illustrated in Fig. 3. The highest blood concentrations were obtained in all ten volunteers within two hours of injection. Total "Elkosin" levels varied from 5.2 to 11.9 mg. per cent (average 8.9 mg. per cent); 92.6 to 100 per cent (average 96.6 per cent) was nonacetylated. At twelve hours total concentrations from 1.4 to 5.2 mg. per cent (average 2.9) was observed of which 76.9 to 95.1 per cent (average 86.5 mg. per cent) was in the free form. After twenty-four hours a range of 0.6 to 2.3 mg. per cent (average 1.3 per cent) was still present, with an average free concentration of 75.3 per cent. At forty-eight hours total "Elkosin" levels from 0.15 to 0.9 mg. per cent were detected, with an average acetylation of only 15 per cent.

Maximum urinary excretion occurred after two, four, six, and eight hours in seven, one, one, and two cases, respectively (Fig. 4). At these times the average peak total urinary concentration was 279 mg. per cent with 92.7 to 100 per cent (average 98.4 per cent) in the free form. After twenty-four hours 45 to 83 per cent (average 69 per cent) of the total injected dose was excreted. Of this amount only 5 to 22.4 per cent was acetylated with an average of only 9.8 per cent. After forty-eight hours over 80 per cent of the drug had been excreted. Specimens saved over this second twenty-four hour period showed an average acetylation of 66 per cent. However, including the latter results in the over-all total of seventy-nine separate determinations done over a forty-eight hour period, an average of 85 per cent of the "Elkosin" excreted was in the free form.

A total of eight spinal fluid specimens was obtained at four hours after injection of the drug while five were taken at eight hours. At the four-hour period an average free level of 1.2 mg. per cent, or approximately 17 per cent, of the average four-hour blood level of 7.0 mg. per cent was observed. After eight hours an average value of 1.3 mg. per cent, or approximately 31 per cent, of the eight-hour blood level of 4.2 mg. per cent was noted.

DISCUSSION

The development of new sulfonamides has been aimed primarily at decreasing possible side effects. It is reasonable to assume that qualitative changes in antibacterial effects cannot be enhanced further. One of the chief objections to sulfonamides has been the potential hazard of renal precipitation. The preliminary studies described herein have demonstrated that "Elkosin" is an exceptionally soluble drug which is readily absorbed from the gastrointestinal tract and rapidly excreted in the urine. It also readily diffuses into cerebrospinal, pleural, and ascitic fluids. Acetylation is remarkably low. Studies in progress here have demonstrated that "Elkosin" was effective in a wide variety of disease processes amenable to sulfonamide therapy.

SUMMARY AND CONCLUSIONS

1. Oral and intravenous administration of a single dose of .05 Gm. per kilogram of "Elkosin" resulted in average peak levels of 10 mg. per cent within two to eight hours with over 95 per cent nonacetylation.

2. Eighty to 90 per cent of the drug was excreted in the urine within forty-eight hours, with an average acetylation of only 13 per cent (seventy-five specimens) and 15.5 per cent (seventy-nine specimens) following oral and intravenous administrations, respectively.

3. After single oral doses, 9.95 and 22.6 per cent of the free blood "Elkosin" levels were observed in spinal fluid four and eight hours after administration, respectively. With continuous oral therapy the spinal fluid concentration averaged 49.6 per cent of that observed in blood. A single intravenous dose yielded 17 and 31 per cent of the four- and eight-hour blood levels, respectively.

4. "Elkosin" diffused readily into pleural and ascitic fluids.

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HUMAN SERUM TRIBUTYRINASE
II. LEVELS IN TUBERCULOSIS

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A NUMBER of human diseases have been reported to be accompanied by marked variations from normal serum tributyrinase levels. Several workers have investigated the lipolytic activity of the serum of patients with tuberculosis. Birath[1] found the "quinine-resistant" lipase concentration increased in the serum of patients with pulmonary tuberculosis. Melchor and Herrero[2] obtained lipase levels inversely proportional to the sedimentation rate, and found that an increase from low levels indicated a favorable prognosis. Paraf, Desbordes, German, and Fournie[3] noted that in advanced tuberculosis with unfavorable prognosis, the rate of hydrolysis of tributyrin was decreased to less than half normal. Lowered serum tributyrinase activity was obtained by Scoz and Guzzi[4] for some tuberculous patients, while others appeared to be in the normal range. According to Machida[5] serum lipase activity is affected not so much by the form and extent of the disease as by the general condition of the patient.

METHODS AND MATERIALS

Samples of blood were obtained from tuberculous patients in three Alberta Hospitals, and essential clinical information was supplied in all cases. Serum was always separated immediately after arrival of the sample in the laboratory. Although there was some delay in obtaining out-of-town specimens, there was rarely any evidence of hemolysis. Previous work indicated that enzyme concentrations were unaltered by samples standing for several days at room temperature. The method for the determination of tributyrinase activity has been described in a previous paper.[6]

RESULTS

Serum tributyrinase activity was determined for 214 tuberculous patients. With a number of these subjects determinations were repeated at intervals in order to correlate the condition of the patient with changes in enzyme activity.

In our preceding paper it is stated that the normal human tributyrinase levels are 300 to 600 units per 100 milliliters. For the studies on tuberculosis, serum tributyrinase values are classified as follows:

Normal	360-550 units per 100 ml.
Low Normal	310-360 units per 100 ml.
Subnormal	210-310 units per 100 ml.
Very Low	below 200 units per 100 ml.

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TABLE I. SERUM TRIBUTYRINASE LEVELS IN NORMAL AND TUBERCULOUS SUBJECTS

		NUMBER OF SUBJECTS	AVERAGE AGE (YRS)	MEAN TRIBUTYRINASE LEVEL IN UNITS/100 ML.	STANDARD DEVIATION	STANDARD ERROR OF THE MEAN
Normal						
Total		280	27 (9-63)	403	±89	±5.0
	Males	174	29	417	±77	±5.8
	Females	106	23	379	±75	±7.4
Tuberculous						
Total		214	34 (9-71)	381	±92	±15.0
	Males	105	38	389	±87	±8.6
	Females	109	29	370	±99	±9.6

Comparison of Means for normal and tubercular subjects:
$t = 2.68$
t at 1% level $= 2.57$

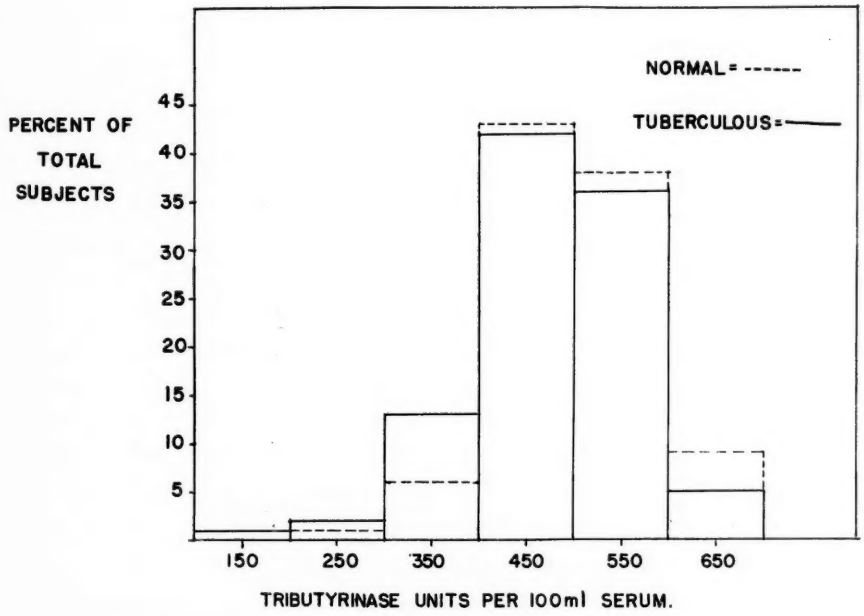

Fig. 1.—Distribution of serum tributyrinase levels in 280 normal and 214 tuberculous subjects.

TABLE II. SERUM TRIBUTYRINASE LEVELS AT DIFFERENT AGE INTERVALS IN TUBERCULAR SUBJECTS

AGE INTERVAL (YEARS)	NUMBER OF SUBJECTS	MEAN TRIBUTYRINASE UNITS PER 100 ML. OF SERUM	STANDARD DEVIATION	STANDARD ERROR OF THE MEAN
15-19	25	382	±90.6	±18.5
20-24	28	379	±99.1	±19.1
25-29	33	370	±84.9	±14.9
30-34	38	373	±118.4	±19.4
35-44	39	390	±92.6	±14.9
45-71	51	381	±88.4	±12.6

TABLE III. PERIODIC SERUM TRIBUTYRINASE LEVELS (UNITS PER 100 ML.) IN PATIENTS WITH SEVERE TUBERCULOSIS

CASE NO.	DIAGNOSIS	AGE	SEX	DETERMINATION NUMBER						INTERVAL BETWEEN DETERMINATIONS
				1	2	3	4	5	6	
1	Pulmonary TB Far advanced Sick	34	F	64	100	208	---	---	---	one week
2	Pulmonary TB Far advanced Sick	32	F	328	332	280	---	---	---	one week
3	TB Empyema Far advanced Very sick	32	F	392	376	420	424	456	456	one month
4	Pulmonary TB Far advanced Sick	36	F	216	192	252	260	232	256	one week
5	Pulmonary TB Far advanced Sick	40	F	152	---	---	---	---	---	--------
6	Pulmonary TB Far advanced Very sick	32	F	48	---	---	---	---	---	--------
7	Moderately advanced Pulmonary TB	17	F	280	360	---	---	---	---	one month
8	Non Pulmonary TB. Left Nephrectomy	22	M	328	584	---	---	---	---	one month
9	Far advanced Pulmonary TB Lobectomy Sick	35	F	268	340	380	---	---	---	one month
10	Pulmonary TB Far advanced Empyema	42	M	308	284	292	---	---	---	one month

A comparison of tributyrinase levels in 280 normal and the group of 214 tuberculous subjects is made in Table I. The mean value for the diseased group is only 5 per cent below the normal, but this difference is statistically significant. There is no significant difference between the means of the female groups, and therefore the difference between normal and tuberculous subjects may be attributed to the males.

Table II indicates that there is no correlation between age and activity of the enzyme in tuberculous persons as there is in normal subjects.

The distribution of serum tributyrinase activities in normal and tuberculous subjects is shown in Fig. 1.

The blood samples obtained were from 214 patients with various forms and degrees of severity of the disease. It was invariably found in mild cases that tributyrinase levels were in the normal range. It was only when the disease was severe that enzyme activity fell to low normal or subnormal values. The results in Table III are typical of our findings with patients suffering from advanced tuberculosis. The initial values are below the normal range, except in Case 3. Such exceptions were rarely found, and it is possible that they may be associated with therapy received by the patient. Gross, Comfort, Mathieson, and Power[7] found that administration of codeine sulfate to patients was followed by elevated values of serum lipase. It might be expected that prolonged treatment with various drugs such as p-aminosalicylic acid and streptomycin and its derivatives would complicate our study.

In general, we found that the condition of the patients with advanced tuberculosis was an important factor in determining the trend in the serum tributyrinase activity of the individual. For example, Cases 1, 4, and 7 showed improvement as a result of treatment, and this was paralleled by a return of lipase levels toward normal. On the other hand, the condition of Case 2 deteriorated, and this was accompanied by decreased enzyme activity.

SUMMARY

Serum tributyrinase levels have been determined in 214 tuberculous patients. This group showed a slight but significantly lower mean value than a group of normal subjects. No correlation was found between age and enzyme activity in tuberculous patients.

Persistent, subnormal values have been found in patients with advanced tuberculosis. Exceptions to this may possibly be attributed to therapeutic measures applied to such patients. Improvement or deterioration in the condition of patients with advanced tuberculosis is reflected in either a rise toward normal or a further fall in serum tributyrinase values.

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THE DETECTION OF OCCULT BLOOD IN FECES INCLUDING OBSERVATIONS ON THE INGESTION OF IRON AND WHOLE BLOOD

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THE detection of occult blood in stools has assumed increasing importance in recent years because of the advent of cancer detection clinics. In order to exclude malignancy of the gastrointestinal tract without resorting to expensive and time-consuming radiographic procedures, examination of the feces for occult blood offers a relatively simple and rapid screening method. The purpose of this study was to investigate the validity and sensitivity of some of the commonly employed reagents used for the detection of occult blood in feces. The study was amplified to include observations on the effect of ingested iron and on the amount of blood which must be shed into the intestinal tract to produce positive reactions.

Van Deen (1864)[1] is generally credited with being the first worker to use guaiac as an indicator for occult blood. The modification of the guaiac test by Weber (1893)[2] is still commonly employed. Schroeder[3] maintained that the amount of guaiac required varied directly with the quantity of blood in the feces. In 1918, Lyle and Curtman[4] introduced a procedure using guaiaconic acid, a constituent of guaiac gum.

Techniques employing the benzidine reagent are more numerous. The use of this compound was first suggested by Adler and Adler.[5] Modifications have been described by Schlesinger and Holst[6] and Lyle, Curtman, and Marshall.[7] Ingham[8] reduced it to a spot test, Roberts[9] to a tablet test, Andrews and Oliver-Gonzáles[10] to a quantitative procedure using a photoelectric instrument, and Groat[11] modified it to a slide procedure.

The most notable contribution was made by Gregersen.[12] He demonstrated that a positive benzidine reaction is influenced by the concentration of benzidine itself. It is perhaps for this reason that the Gregersen technique, which employs benzidine in a concentration necessary to detect pathologic quantities of blood, is regarded as the most reliable procedure.[13, 14] Experiments in this laboratory demonstrated that a reaction which is indisputably negative can become strongly positive without changing the quantity of feces when the concentration of benzidine is changed from 5 mg. to 10 mg. Burger,[15] Leiboff,[16] and Bing and Baker[17] had previously noted the effect of the concentration of benzidine on the intensity of the reaction.

Other methods for determining the presence of occult blood involved the detection of "soluble albumin"[18, 19] or hematin crystals.[20, 21] Some were based on immunologic principles[22, 23] or required the use of a spectroscope.[24-28]

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Among the other indicators used were ferrocyanide, 3-aminophthalic acid hydrazide,[29, 30] paraphenylendiaminchlorhydrate,[31] pyramidon,[32, 33] aloin,[34] phenolphthalin,[35-38] and orthotolidin.[39-41] The last two reagents, as well as benzidine and guaiac, have been investigated in this study.

Orthotolidin was introduced as an indicator for blood by Ruttan and Hardisty (1912).[39] Stone and Burke[40] recommended its use for the detection of blood in urine. Adler and Adler[5] preferred the use of its analogue, benzidine.

Although phenolphthalin appears to be superior to benzidine, it has not received adequate attention. This is difficult to explain since phenolphthalein possesses several decided advantages. The reaction results from the oxidation of this reagent to phenolphthalein (presumably by the blood oxidases) which is pink in an alkaline medium. The reaction is instantaneous and unequivocal. The amount of reagent used is kept constant, thus insuring uniformity in results. Moreover, the sensitivity of phenolphthalein does not vary with different lots as with benzidine or guaiac. It forms a stable solution, but care must be taken to keep the reagent bottle well stoppered.

In view of the many methods available for the detection of occult blood and of the different sensitivities of the reagents used, it is understandable why discrepancies are encountered frequently. Standardization of technique would be of some assistance in lessening the confusion. This may be questioned, however, in the case of guaiac or benzidine, since their sensitivities are known to vary with different lots. Schumm and Westphal[42] showed that one sample of benzidine may be ten times as sensitive as another.

In the present study, the sensitivities of four indicators (orthotolidin, benzidine, phenolphthalein, and guaiac) were compared. The effect of the ingestion of iron and whole blood was also investigated. Two kinds of excreta, namely, stool and urine, were studied. Possible sources of error were evaluated by investigating a number of interfering substances, most of which were inorganic in nature.

LABORATORY METHODS

The stool emulsions were prepared as follows: the specimen of feces was rendered as homogeneous as possible by means of a stirring rod and a small portion, about the size of an overgrown pea, was removed with the stirring rod and added to a tube containing approximately 4 ml. of water. The mixture was heated by passing the test tube several times over the flame of a Bunsen burner and allowed to cool to room temperature.* One milliliter of this cooled mixture was used for the determinations. Measurements were exact and constant to insure comparable results. With the orthotolidin test (see description below), this was not possible. Some of the stool on the stirring rod was used to make a watery paste. A thin film of this paste was rubbed on the filter paper and the orthotolidin tablet placed on the moist area. Controls, consisting in each instance of the required reagents, were carried out simultaneously. When the results were equivocal, an additional control, consisting of the specimen and all the reagents except hydrogen peroxide, was used. The activity of hydrogen peroxide was checked with potassium dichromate in concentrated sulfuric acid. All glassware was chemically clean.

*This step, though commonly employed, is unnecessary. In data obtained in this study (not tabulated), heated or unheated stool emulsions gave the same intensity of reaction with the various indicators used.

The orthotolidin test was carried out according to the recent "tablet method" outlined by the manufacturers of the product.* A portion of the watery paste of stool (as described above) was placed on a square of filter paper (supplied with the tablets) and the tablet was placed in the center of the moist area. Two drops of water were placed on the top of the tablet and allowed to roll down the sides. Exactly two minutes were allowed for the reaction. The presence of a blue color on the filter paper around the tablet was considered positive. Positive reactions were graded according to the intensity of color (from ± to ++++).

For the benzidine test, 1 ml. of stool emulsion was mixed with 1.5 ml. of glacial acetic acid containing a pinch† of benzidine in a test tube. Five drops of 3 per cent hydrogen peroxide were added dropwise with shaking. Precisely two minutes were allowed for the reaction. A blue or green color was considered positive. The intensity of the reaction was based on the speed and depth of color development.

The guaiac test was performed as follows: to 2 ml. of glacial acetic acid in a test tube, exactly 1 ml. of the stool emulsion was added. To this was added 2 ml. of ether and 2 ml. of alcoholic guaiac solution (1 Gm. in 60 ml. of 85 per cent ethyl alcohol). Five drops of 3 per cent hydrogen peroxide were added dropwise with shaking, and the results read immediatly. A green or blue color was considered positive. The rapidity and depth of color development determined the intensity of the reaction.

The phenolphthalein test of Meyer was followed according to the directions described by Gettler and Kaye.[41] The reagent was prepared by dissolving 20 Gm. of sodium hydroxide in 200 ml. of distilled water in a 500 ml. Erlenmeyer flask equipped with a reflux condenser. One gram of phenolphthalein was added and the flask agitated to dissolve the material. Twenty grams of granulated zinc were added. The mixture was refluxed slowly (from two to six hours) until the red color of the alkaline phenolphthalein disappeared. A colorless or faintly yellow solution remained. The solution (with the zinc granules) was transferred to a rubber-stoppered brown bottle and stored in a refrigerator. The test for occult blood was carried out as follows: 1 ml. of the stool emulsion and 5 drops of the phenolphthalein reagent were placed in a test tube. Three drops of 3 per cent hydrogen peroxide were added dropwise and the contents of the test tube agitated after the addition of each drop. A positive reaction was indicated by the development of a pink to red color. Results were read immediately because of the rapid fading of the color. The intensity of the reaction was graded by the rapidity and depth of the color development.

SUBJECTS AND MATERIALS

Eight normal female laboratory technicians (ages 20 to 28 years) were the subjects of some of the experiments reported here. These volunteers were used to study the effect of ingested iron and whole blood. These technicians were placed on a meat-free and fish-free diet which was maintained for the duration of the experiments. Tablets of ferrous sulfate‡ were taken orally over a period of three to five days (7.8 to 19.4 Gm.). Stools were examined daily beginning twenty-four hours after the first dose of iron.

The subjects for the blood studies were given an initial dose of 0.5 ml. of whole blood. Increments were given when blood from the previous ingestion had proved undetectable by the chemical methods described above. Each subject ingested her own blood which was measured into individual gelatin capsules as it was withdrawn from her vein.

For the sensitivity studies on blood added to feces, an entire specimen of stool, negative for occult blood by the procedures described above, was mixed with approximately 500 ml. of distilled water. Mixing was accomplished by a shaking machine. The undissolved particles were allowed to settle to the bottom of the bottle, and the supernatant fluid was

*This tablet is commercially known as Hematest and was supplied by the Ames Company, Inc., Elkhart, Ind.

†This is the procedure routinely employed in the Laboratory of Pathological Chemistry of this institution. A pinch is approximately equivalent in volume to a drop of water delivered by a 1 ml. Ostwald-Folin pipette.

‡Feosol tablets manufactured by Smith, Kline & French Laboratories, Philadelphia, Pa.

mixed with blood in various dilutions. One milliliter of these dilutions was used for all the determinations except orthotolidin; only one or two drops of the respective dilutions were used with this indicator.

For sensitivity studies on blood added to urine, the dilutions used are indicated in Table III. The urine consisted of pooled centrifuged specimens which were free from blood on microscopic and chemical examination. One milliliter of each dilution was used for the individual tests except for the orthotolidin procedure when one or two drops were employed.

The specimens of feces for the comparative sensitivity studies were obtained from the wards of this institution; seventy in all were studied. No attempt was made to correlate the laboratory findings with clinical disorders.

A number of substances which might produce false positive reactions for blood were also tested. These are indicated in Table VIII. Hemoglobin was included in this series for comparative purposes. No substrate of stool was used in these experiments; the compounds were added directly to the indicator.

RESULTS

Stools from three normal subjects, E. S., D. G., and J. W., who had ingested iron, gave negative guaiac, phenolphthalein, and benzidine reactions. Only faintly positive reactions were noted with orthotolidin. Subject E. S. was given 0.65 Gm. of ferrous sulfate four times a day and had received a total of 7.8 Gm. at the time of the last stool examination. D. G. received 0.97 Gm. of ferrous sulfate four times a day; total intake at the time of the last examination was 11.6 Gm. J. W. was given daily increments of ferrous sulfate and the results represented the cumulative effect of 19.4 Gm. over a period of five days. These results are not tabulated because uniformly negative reactions were obtained except for the faintly positive reactions with orthotolidin.

The results obtained with ingested blood are indicated in Tables I and II. Table I shows the findings on subjects N. E. and E. S. who were on a meat-free and fish-free diet. It may be noted that orthotolidin gives a positive reaction following the ingestion of 1 ml. of blood, while benzidine and phenolphthalin require 3.5 ml. for faint reactions and 5 ml. for stronger reactions. Not until 20 ml. of blood were ingested was any positive reaction with guaiac noted. This table illustrates the insensitivity of the guaiac reaction for the determination of occult blood in stool following the ingestion of whole blood by subjects on a *restricted* (meat-free and fish-free) diet.

Table II reveals the results obtained with guaiac on three subjects on an *unrestricted* diet after the ingestion of whole blood. It may be noted that under these circumstances, the guaiac test detects from 2 to 3 ml. of ingested blood, the intensity of the reaction being no less than that produced by 20 or 25 ml. on a meat-free and fish-free diet. The amount of stool used in both experiments (Tables I and II) was approximately the same, namely, 60 to 75 milligrams.

It has long been known that ingested blood may not be completely evacuated within twenty-four hours. This is illustrated in Table I. It can be seen that sixty-nine hours or more are required for the complete evacuation of 3.8 to 20 ml. of blood. It seems reasonable that the number of days required for complete evacuation of blood is somewhat governed by the amount of blood ingested. Additional evidence for this may be found in the work of Schiff and

TABLE I. THE ESTIMATION OF OCCULT BLOOD IN FECES USING VARIOUS REAGENTS FOLLOWING THE INGESTION OF WHOLE BLOOD (NORMAL SUBJECTS ON A MEAT-FREE AND FISH-FREE DIET)

SUBJECT	ML. WHOLE BLOOD INGESTED	TIME STOOL PASSED (HOURS AFTER INGESTION)	ORTHO-TOLIDIN	BENZIDINE	PHENOL-PHTHALIN	GUAIAC
N. E.	0.5	24	±	0	0	0
	1.0	22	++	0	0	0
	2.0	20	++	0	0	0
	3.5	21	±	±	±	0
		48	±	*	±	0
	3.8	17	±	0	0	0
		45	++	++	++	0
		66	++++	++++	++++	0
	4.5	2	0	0	0	0
		19.5	±	*	0	0
		48	++	±	++	0
	5.5	21	++	+++	++	0
		49	++	++	++	0
		72	0	0	0	0
	7.0	17	++	+++	++	0
		43.5	++	++++	++++	0
		67	±	+	++	0
		77	++	0	0	0
	9.0	21.5	++	++++	++++	0
	10.0	18.5	++++	+++	++++	0
		43	++++	+	++	0
		66.5	±	0	0	0
	12.0	23	++++	+++	++++	0
		46.5	++	+++	++++	0
		73	++	0	±	0
E. S.	15.0	18.5	±	*	±	0
		25	++	++	++	0
N. E.	20.0	24	++	+++	++++	±
		44.5	++	+++	++++	0
		69	0	0	0	0
E. S.	25.0	18	0	0	0	0
		24	++++	++++	++++	±
N. E.	30.0	24	++++	++++	++++	±
		44.5	++++	++++	++++	±

*Change of color to dark brown but not green or blue.

TABLE II. THE ESTIMATION OF OCCULT BLOOD IN FECES USING THE GUAIAC REAGENT FOLLOWING THE INGESTION OF WHOLE BLOOD (NORMAL SUBJECTS ON AN UNRESTRICTED DIET)

SUBJECT	ML. WHOLE BLOOD INGESTED	TIME STOOL PASSED (HOURS AFTER INGESTION)	GUAIAC REACTION
M. G.	2	22	0
		68	±
E. F.	2	22.3	*
		46.5	0
		72.5	0
E. F.	3	21	*
		44	±
N. E.	3	21	±
		42	±

*Doubtful.

TABLE III. SENSITIVITY OF VARIOUS REAGENTS FOR THE DETECTION OF BLOOD ADDED DIRECTLY TO URINE

DILUTION	ORTHOTOLIDIN	BENZIDINE	PHENOLPHTHALIN	GUAIAC
1:100	++++	++++	++++	++
1:200	++++	++++	++++	±
1:300	++++	++++	++++	±
1:400	++++	+++	++	±
1:500	++++	+++	++	±
1:600	++++	++	++	±
1:700	++++	++	++	±
1:800	++++	±	++	±
1:900	++++	±	++	±
1:1000	++++	±	±	±
1:1100	++++	±	±	±
1:1200	++++	±	±	0
1:1300	++++	±	±	0
1:1400	++++	±	±	0
1:1500	++++	±	±	0
1:1600	++++	±	0	0
1:1700	++++	±	0	0
1:1800	++++	±	0	0
1:1900	++++	±	0	0
1:2000	++++	0	0	
1:4000	++	0	0	
1:5000	++	0	0	
1:10,000	++	0	0	
1:20,000	±	0	0	
1:50,000	±	0	0	
1:100,000	0	0	0	

TABLE IV. SENSITIVITY OF VARIOUS REAGENTS FOR THE DETECTION OF BLOOD ADDED DIRECTLY TO FECES

DILUTION	BENZIDINE	PHENOLPHTHALIN	GUAIAC
1:100	++++	++++	++
1:200	++++	++	±
1:300	++++	++	±
1:400	++++	++	±
1:500	++++	±	±
1:600	++++	±	±
1:700	++++	±	±
1:800	++++	±	±
1:900	++++	±	±
1:1000	++++	±	±
1:1100	++++	±	±
1:1200	++++	±	±
1:1300	++++	±	±
1:1400	++++	±	0
1:1500	++++	0	0
1:2000	++++	0	0
1:4000	++++	0	0
1:5000	+++	0	0
1:10,000	+	0	0
1:20,000	+	0	0

his co-workers.[43] They demonstrated that the stools from a patient ingesting 250 ml. of blood may give positive reactions for blood for as long as ten days.

The sensitivity of indicators for blood added directly to urine and feces is shown in Tables III and IV, respectively. Orthotolidin is the most sensitive of the indicators used. Benzidine, phenolphthalin, and guaiac follow in that order. The influence of the medium on which these tests are carried out is worthy of note. The inhibitory effect of urine itself has already been noted by

Larsen[44] and by Trainer.[45] Even the reputed sensitivity of benzidine fails in this medium. It is not uncommon to observe a negative benzidine test when thirty to forty red blood cells per high power field are seen microscopically. For this reason, the microscope is a more dependable means for detecting hematuria.

TABLE V. THE DETECTION OF OCCULT BLOOD IN CLINICAL SPECIMENS OF STOOL USING VARIOUS INDICATORS

SPECIMEN NO.	ORTHOTOLIDIN	BENZIDINE	GUAIAC
1	++	++++	0
2	++++	±	0
3	++++	0	0
4	±	0	0
5	++++	++++	±
6	0	0	0
7	0	±	0
8	++	0	0
9	±	±	0
10	++++	0	0
11	++	±	0
12	±	0	0
13	++++	0	0
14	++	++++	±
Total positive	12	7	2

TABLE VI. THE DETECTION OF OCCULT BLOOD IN CLINICAL SPECIMENS OF STOOL USING VARIOUS INDICATORS

SPECIMEN NO.	ORTHOTOLIDIN	BENZIDINE	PHENOLPHTHALIN
15	++++	++++	++
16	++++	++++	++
17	0	0	0
18	++	++++	++++
19	±	0	0
20	++	±	±
21	0	0	0
22	±	+++	0
23	±	++	0
24	±	0	0
25	++	±	±
26	++	++++	++
27	++	±	±
28	±	+	±
29	++	+	++
30	±	±	++
31	±	0	0
32	±	±	±
33	±	0	0
34	±	0	0
Total positive	18	13	11

In order to conserve space, Tables V and VI indicate only some of the results obtained with clinical specimens of stool from patients on unrestricted diets. Table V compares the orthotolidin, benzidine, and guaiac reactions. It will be noted that twelve of the fourteen specimens gave a positive reaction with the orthotolidin test, seven a positive reaction with the benzidine test, and only two with the guaiac procedure. The relative sensitivity of these indicators corroborates the findings described elsewhere in this paper. Table

VI compares the orthotolidin, benzidine, and phenolphthalin procedures as applied to stool specimens. Eighteen of the twenty specimens gave a positive reaction with the orthotolidin test, thirteen with the benzidine reagent and eleven with the phenolphthalin procedure. It will be noted that there is somewhat better agreement between the orthotolidin and bendizine reactions in Table VI as compared with Table V. The only difference in the two series of experiments was the use of a new supply of orthotolidin tablets for the studies reported in Table VI. One suspects that the sensitivity of this indicator may vary with different lots.

DISCUSSION

The Effect of Ingested Iron.—The earliest record in the English language (1905) on this subject is that of Steele and Butt.[46] These observers examined the stools of patients taking 30 grains (1.8 Gm.) of ferric carbonate daily; the specimens failed to give positive reactions for blood with the guaiac and aloin reagents. Ovoferrin, 1½ ounces (45 Gm.) daily, was also given; these stools gave negative reactions for blood.

Ogilvie[47] studied two patients who had taken iron continuously for more than three months. The stools of these subjects gave persistently negative reactions to the pyramidon and Gregersen benzidine tests.

Conflicting results have been presented by other investigators. Burger[15] reported that iron medication rendered the benzidine test positive, while the guaiac test remained negative. On the other hand, Johnson and Oliver[48] found that iron medication rendered the guaiac test positive while the benzidine test remained negative. They assumed that these false-positive reactions were due to the tannin in guaiac combining with iron producing a blue iron tannate.

A more extensive study was reported by Schwartz and Vil.[49] These workers used nineteen subjects and observed the effect of therapeutic doses of several iron preparations on the benzidine reaction. Of eighty-one stool examinations, only one positive reaction was obtained.

In spite of these conflicting reports, it might be stated categorically that the ingestion of ferrous sulfate does not invalidate the results obtained with these reagents for the estimation of occult blood. It was found, rather, that the addition of hydrogen peroxide in the benzidine and phenolphthalin reactions cleared the suspensions of feces from subjects taking concentrated doses of iron. Slightly positive reactions may occur with orthotolidin. However, the significance of this finding may be doubted since orthotolidin not infrequently produces positive reactions with stools from apparently normal individuals on meat-free and fish-free diets.

The Effect of Ingested Blood.—The sensitivity of an indicator has often been evaluated by adding blood to stool or urine in vitro. While this method may be a means of calculating the amount of blood necessary to produce a positive reaction, it cannot be extended to blood which has been shed internally unless bleeding occurs low in the tract. The experiments of Bramkamp and Banning[50] showed that 0.08 ml. of blood was detectable when fresh blood was hemolyzed and mixed directly with feces. They found, however, that ninety

times this amount was required in order to obtain a positive reaction when blood was taken orally. These observers demonstrated that the intensity of the reaction between blood and benzidine was reduced if the blood was digested by pancreatic juice prior to its addition to benzidine.

Examination of stools from subjects ingesting blood, therefore, more nearly approaches the situation of a patient who is bleeding into the gastrointestinal tract. A number of workers have investigated this problem; their findings are shown in Table VII.

TABLE VII. SURVEY OF THE LITERATURE ON THE AMOUNT OF INGESTED BLOOD REQUIRED TO PRODUCE POSITIVE REACTIONS FOR OCCULT BLOOD IN STOOL USING VARIOUS PROCEDURES

OBSERVER	GUAIAC (ml.)	BENZIDINE (ml.)	GUAICONIC ACID (ml.)	PHENOL-PHTHALIN (ml.)	ORTHO-TOLIDIN (ml.)	SPECTRO-SCOPIC (ml.)
Weber[2]	3					
Rossel[51] (1903)	5-10					
Cowie[52] (1907)	0.5					
White[53] (1907)	3					
Abrahams[54] (1920)		1				
Reimann[14] (1922)	2-4	1	more than 3			
Bell[55] (1923)	1	1		1		
Kiefer*[56] (1934)						
Water extracts		5			2-4.5	2-3
Ethereal extracts		1-3			1	
Present report:						
Meat-free and fish-free diet	20	3.5		3.5	1	
Unrestricted diet	2-3					

*This observer based his results on the amount of blood ingested per day.

It is difficult to evaluate the results presented in this table. The significance of small differences may be questioned since these tests are influenced by a multiplicity of factors, viz., quality of reagents, agitation, time allotted for reaction, antecedent diet, amount of specimen or quantity of indicator used, and the type of extraction employed. This last consideration is demonstrated by the results of Kiefer.[56]

Table VII indicates further that the ingestion of much larger quantities of blood are necessary to produce a positive reaction with guaiac in the present experiments as compared with those of earlier workers. This may be ascribed to the fact that smaller quantities of stool were employed in the present studies compared to quantities of 3 to 5 Gm. or more used by previous investigators.

Interfering Substances.—It has long been recognized that substances unrelated to blood may duplicate the reactions between blood and reagents. The results presented in Table VIII are not unlike those of Gettler and Kaye.[41] These investigators included sulfonamides, salicylates, bismuth subnitrate, and bromides in their studies and found that these substances failed to give positive reactions with phenolphthalin, benzidine, guaiac, or orthotolidin. The possible confusion that may be introduced by interfering substances has been reported by Abrahams[57] who noted that a patient with no clinical signs of malignancy excreted stools which were persistently positive for blood by the benzidine indicator. By chance, it was discovered that the patient was ingesting a self-prescribed dose of potassium permanganate daily.

TABLE VIII. THE ACTION OF VARIOUS SUBSTANCES ADDED DIRECTLY TO REAGENTS USED FOR THE DETECTION OF OCCULT BLOOD

SUBSTANCE	ORTHOTOLIDIN (POSITIVE REACTION INDICATED BY BLUE COLOR)	BENZIDINE (POSITIVE REACTION INDICATED BY BLUE OR GREEN COLOR)	PHENOLPHTHALIN (POSITIVE REACTION INDICATED BY PINK COLOR)
$CuSO_4$ (20%)	±	Color interference	++
$FeCl_2$ (10%)	±	Reddish-brown color but not green	Color interference
$KMNO_4$ (0.005 N)	±	Benzidine solution becomes green upon the addition of $KMNO_4$	Color interference
KI (10%)	++++	Orange-brown color but not blue	0
Sodium nitro-ferricyanide (saturated solution)	++	±	0
Colloidal Iron	±	0	0
$HgCl_2$ (saturated solution)	0	±	Precipitation occurs but is not pink
Hemoglobin	++++	++++	++++
Pus	±	±	0

Diet.—Most workers agree that a diet devoid of meat and fish for at least three days prior to the examination of the stool is a necessary prerequisite in order to obtain valid results. In the present study when the diets of the normal subjects were unrestricted, the orthotolidin, benzidine, and phenolphthalin tests for occult blood in the stool were strongly positive. With one exception, the guaiac test was consistently negative. When the normal subjects were placed on a meat-free and fish-free diet, the reactions to the four indicators were negative within seventy hours. This evidence strongly supports the view that a restricted diet is necessary for clinical evaluation; the guaiac test, as described in this paper, is an exception to this rule. White[58] claimed that the Weber (guaiac) test often gave negative results when one-quarter to one-half pound of meat was ingested (extent to which meat was cooked not stated).

Due to the chemical similarity between chlorophyll and hemin, it has been suspected that chlorophyll may interfere with tests for occult blood. While it may be a source of confusion spectroscopically, the ingestion of chlorophyll appears to have no effect on the tests used in the present study. The subjects in this investigation on a meat-free and fish-free diet were permitted usual portions of green vegetables including spinach, beans, and broccoli. Benzidine, guaiac, and phenolphthalin indicators failed to give positive reactions. At times, the orthotolidin test was positive but due to the marked sensitivity of this reagent, it cannot be stated that this was due to chlorophyll.

Gum-Bleeding.—It is probable that mild gum-bleeding such as produced by brushing the teeth does not affect the benzidine, phenolphthalin or guaiac tests; it may interfere with the orthotolidin procedure. Restrictions concerning brushing the teeth seem to be unnecessary. One volunteer in this study (on a meat-free and fish-free diet for five days) reported gum-bleeding; stools from

this subject were checked on the following three days. The guaiac, benzidine and phenolphthalin tests were persistently negative.

SUMMARY AND CONCLUSIONS

The following reagents for the detection of occult blood in stool and urine were compared: orthotolidin, benzidine, phenolphthalin, and guaiac. Orthotolidin proved to be the most sensitive indicator; benzidine, phenolphthalin, and guaiac followed in that order. The sensitivity of orthotolidin is considered misleading rather than advantageous, since false-positive reactions with this indicator are common. False-positive reactions with benzidine are frequent if the quantity of benzidine used is not restricted.

A meat-free and fish-free diet for seventy-two hours prior to examination of stool for occult blood is essential for the orthotolidin, benzidine, and phenolphthalin tests; procedures using the guaiac indicator require no such dietary restriction.

The ingestion of ferrous sulfate does not produce false-positive reactions with these reagents; orthotolidin is a possible exception.

The amount of blood which must be ingested to give positive reactions with these indicators by normal subjects on a meat-free and fish-free diet has been determined: orthotolidin 1 ml., benzidine 3.5 ml., phenolphthalin 3.5 ml., and guaiac 20 ml. Normal subjects on an unrestricted diet will show faintly positive reactions to guaiac after the ingestion of 2 to 3 ml. of blood.

Mild gum-bleeding, such as produced by brushing the teeth, is not sufficient to render these procedures positive. Foods containing chlorophyll likewise do not invalidate these procedures.

The detection of occult blood in urine by these reagents is not recommended since urine itself inhibits the reaction between blood and the indicators.

A number of substances unrelated to blood added directly to the reagents may produce color interference or false-positive reactions; these are indicated in the text.

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NORMAL AND ABNORMAL VARIATIONS AND CLINICAL SIGNIFICANCE OF THE ONE-MINUTE AND TOTAL SERUM BILIRUBIN DETERMINATIONS

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THE studies of van den Bergh and his associates[1, 2] aroused great interest in the serum bilirubin and gave impetus to many studies of the nature of what has generally come to be known as the van den Bergh reaction.[3] This is actually the diazo reaction, first described by Ehrlich,[4] and studied later in more detail by Pröscher[5] and by Orndorf and Teeple.[6] It is curious that while this color reaction for bilirubin was known for many years, and while the presence of bilirubin in the serum had long been recognized, the application was not made until van den Bergh's work, first published in detail in 1918.

A brief review of the important findings of van den Bergh and his coworkers is appropriate, since some of their observations appear to have been lost sight of in more recent years. They recognized two distinct types of reactions given by two groups of body fluids or serums. The first group, including diluted bile or the blood serum from patients with "obstructive jaundice," exhibited a "direct" diazo reaction which they emphasized was prompt or even immediate in character, "in a few seconds, at most 30." In the second group, on the contrary, the direct reaction was slow in its development. This group included hemorrhagic fluids from body cavities or blood serums from hemolytic jaundice or pernicious anemia patients. "It required 2, 3, 4 minutes or longer before the reaction began to be definite and still longer before it had reached its greatest intensity. If one repeated the test with the addition of alcohol, then the reaction was maximal almost in the same moment."[3] The addition of alcohol constituted the "indirect" reaction. Many serums from patients with hemolytic jaundice and pernicious anemia, as well as normal horse serum and hemorrhagic fluids from body cavities, exhibited either an indirect reaction only, or a direct reaction that was long-delayed in its development.

In the period shortly after van den Bergh's publications, other investigators gave emphasis to the so-called "biphasic" reaction, which meant that some of the color developed promptly or almost at once, while some developed slowly.[7, 8] This term, however, has not met with any general acceptance mainly

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because the earlier belief has not been borne out, that recognition of the "biphasic prompt" as contrasted with the "biphasic delayed" type of reaction would serve to distinguish between extrahepatic obstructive jaundice on the one hand and parenchymal or diffuse hepatic jaundice on the other. Nevertheless, nearly all jaundiced serums exhibit biphasic reactions in some degree and, when the proportion of the delayed reacting component is considerable, the likelihood of some form of retention jaundice is greatly increased.

The fundamental basis for the difference in the types of van den Bergh reaction has not been established. Various possibilities have been considered in detail in previous reviews, and the evidence for these need not be repeated at present. It will suffice to say that when this subject was last considered by Watson,[9] the hypotheses that the indirect or delayed reacting type was represented by bilirubinglobin and the prompt direct by sodium bilirubinate appeared to be most reasonable. In the interval papers have appeared which question the existence of different types of reaction. No new facts have been presented, though the old idea that bile salts are responsible for the prompt direct reaction has been revived by Baumgärtel[10] and Kühn.[11] The latter, however, has confirmed the observation which was van den Bergh's basis for rejecting the idea, i.e., that the concentration of bile salts required to accelerate the reaction is much greater than one encounters in jaundiced serums. To this may be added the fact that in some cases of regurgitation jaundice due to severe liver disease, large amounts of prompt reacting bilirubin are observed in the serum in the absence of any increase of bile salts.

While it is clear that the mechanism of the prompt direct and delayed or indirect van den Bergh reactions requires further study, one might have assumed, both from the clarity of van den Bergh's monograph and the many subsequent papers attesting to the clinical value of the different qualitative reactions, that a fractional quantitative procedure would have been both logical and generally acceptable. It may be presumed that van den Bergh did not attempt this simply because of the lack of a sufficiently sensitive colorimeter.

In 1945 a simple modification of the Malloy-Evelyn technique[12] for the fractional serum bilirubin determination was reported by Ducci and Watson.[13] In this paper it was suggested that the prompt direct reacting serum bilirubin be determined one minute after the addition of the diazo reagent to the diluted serum. This was quite in accord with van den Bergh's emphasis on the difference between the immediate direct, developing mainly within the first thirty seconds, and the delayed direct, developing slowly after two, three, or more minutes. Later, Watson[9] studied the direct reaction of diluted serums at one-minute intervals from the time of mixture up to fifteen minutes. The character of the reaction curve was believed to be consistent with the presence of two components of the serum bilirubin, one reacting much more slowly because of some difference in its physical-chemical state. Similar data had already been described by others.[14-16]

Employing the fractional bilirubin determination in the above manner, data were obtained for a moderate number of normal individuals and for vari-

ous patients with different types of jaundice.[9] From this it was clear that the amount of prompt reacting (one-minute) bilirubin in the normal serum was extremely small, usually less than 0.15 mg. per 100 ml., and in that series always less than 0.20 mg. per 100 milliliters. It was evident, too, that the amount of each fraction was not necessarily related to the total amount, since in certain instances of pure retention jaundice the increase of the one-minute fraction was only slight, while that of the total bilirubin was considerable. Conversely, cases were observed in which the total bilirubin was but slightly or not at all increased, yet in which there was significant elevation of the prompt reacting fraction.

Gray and Whidborne,[17] and more recently Klatskin and Drill,[18] have described results which led them to deny the significance of the one-minute or prompt direct reacting serum bilirubin. The former interpret the shape of the direct reacting curve as a consequence of the failure of Beer's law above concentrations of 1.6 mg. per 100 ml. of final solution. Their conclusions, however, are not applicable to results with quantitative photometric methods.

Klatskin and Drill conclude that the one-minute determination is of no value because (a) "over a wide range of values there is a more or less direct relationship between the one-minute and total serum bilirubin levels," (b) "there is no significant difference in either the concentrations of one-minute bilirubin or in the one-minute: total bilirubin ratios in extrahepatic obstruction, cirrhosis, hepatitis, and a variety of miscellaneous conditions," and (c) the low one-minute: total bilirubin ratios of four patients with hemolytic jaundice "fall within the lower limits of the range observed in other types of jaundice, and so are of little value in differentiating this type of jaundice from the others." Aside from the facts that the data on which their conclusions are based appear to be excessively variable, that no tests of significance of differences between disease groups are presented, and that four undefined hemolytic jaundice cases comprise a rather tenuous sample from which to generalize, it is not clear how any of the results cited preclude the valid use of the one-minute determination.

The studies to be presented in this paper have two primary objectives: first, to define quantitatively normal and abnormal variations of the serum bilirubin determinations, and second, to evaluate the clinical significance of the one-minute bilirubin.

Included under the first objective are: (a) delineation of the normal limits of the one-minute and total serum bilirubin tests; (b) analysis of the comparative contributions of technical, physiological, and individual variations to the over-all variability of the tests, and measurement of the test-retest reliability; and (c) determination of the correlation of one-minute and total bilirubin values in patients with parenchymal liver disease or biliary obstruction.

Included under the second objective are: (d) description of the variations in the color development curves of direct reacting bilirubin in serums of jaundiced patients, and definition of the basis for selecting the one-minute determination as a quantitative measure of the prompt direct bilirubin; (e) observa-

tion of the comparative incidence of abnormal values with the one-minute and total bilirubin tests in various liver diseases; and (f) evaluation of the discriminative value of the bilirubin ratio ($1'/T \times 100$).

METHODS

Case Material and Procedures Used.—

(a) *In defining normal limits of the one-minute serum bilirubin (1'B) and total serum bilirubin (TB) tests:* The norm group consisted of 719 healthy males called in for examination during a veteran's follow-up survey. Half of the group had viral hepatitis approximately five years previously; the others were their controls. None of the subjects had any evidence of liver disease after exhaustive study, including careful examination and a battery of eleven liver function tests. The 1'B and the TB frequency distributions for these two subsamples were nearly identical. Their means and standard deviations corresponded closely, and significance tests for differences between the means of these subsamples yielded critical ratios of zero and 0.46 for the 1'B and TB comparisons, respectively. Being indistinguishable on the basis of their bilirubin values, the two subsamples were combined to form the norm group. The average age of this group was 30.3 ± 5.2 years; average weight, 162 ± 24 pounds; and average surface area, 1.86 ± 0.14 square meters. Frequency and cumulative frequency distributions were plotted for the 1'B and TB data.

(b) *In analyzing the over-all variation of these tests into component parts and in studying test-retest reliability:* Analyses were carried out separately for normal and abnormal subjects, tests were performed on each of two consecutive days, and duplicate determinations were made with each day's specimen. For each test there were thus four sets of determinations on each of two groups of patients. The normal subjects were thirty-two patients from the neurology and psychiatry wards of the Veterans Administration Hospital, who had no evidence of liver disease. The abnormal subjects were consecutive patients with various liver diseases, who had abnormal bilirubin values. Because we were interested primarily in the variation within the abnormal range of these tests and had not established exact limits of normal at the time, 1'B values of at least 0.3 mg. per cent and TB values of at least 1.7 mg. per cent on one determination were required for inclusion in the sample. There were thirty-one abnormal cases satisfying the criterion for the 1'B test, twenty-three cases for the TB test.

In the normal subjects the maximum range of individual 1'B values was 0.02 to 0.24 mg. per cent, and the range of mean values was 0.10 to 0.14 mg. per cent. In the abnormal subjects the corresponding ranges were 0.13 to 24.95 mg. per cent and 3.61 to 3.67 mg. per cent, respectively. The maximum range of individual TB values in the normal subjects was 0.1 to 1.5 mg. per cent, and the range of mean values was 0.45 to 0.53 mg. per cent. Corresponding ranges in the abnormal subjects were 1.6 to 44.5 mg. per cent and 8.31 to 8.74 mg. per cent, respectively.

Since tests were performed in duplicate on each of two days for each group of patients, there were three criteria for subdividing the total variation on these tests into component parts, viz., variation among individuals, variation from day to day, and variation between duplicates. Analysis of the variation of a given test into these main components together with their interactions takes the following form (using the 1'B in normal subjects as an example):

Source of variation	*df*
Days (Da)	1
Individuals (I)	31
Duplicates (Du)	1
Days × Individuals (Da × I)	31
Days × Duplicates (Da × Du)	1
Individuals × Duplicates (I × Du)	31
Days × Individuals × Duplicates (Da × I × Du)	31
Total	127

The second order interaction (Da × I × Du) is used as the estimate of error for initial comparisons. One can combine all degrees of freedom and corresponding sums of squares for comparisons having no significant effect to form a pooled estimate of the error mean square from which the standard deviation may be obtained.

Test-retest correlations were used as measures of the reliability of the tests. Duplicates for each day were averaged before the correlations were determined. Mean per cent differences between test and retest were also calculated after averaging the duplicates for each day. In calculating per cent differences the larger test score was taken as the reference value.

These analyses were carried out for each of the four sets of data, viz., 1'B, normal subjects; 1'B abnormal subjects; TB, normal subjects; and TB, abnormal subjects.

(c) *In determining the correlation of 1'B and TB values in regurgitation jaundice:* A plot was made on a log log scale of data from 555 cases with various diseases affecting the liver. Cases with cirrhosis, viral hepatitis, obstructive jaundice, congestive failure, and miscellaneous diseases were represented in the same proportions as in the parent sample of 614 cases considered in section (f) below.

Inspection of the scatter diagram revealed three overlapping regions displaying different degrees of association between the 1'B and TB. The correlation of the two variables was therefore observed within each region.

(d) *In describing variations in the color development curves of direct reacting bilirubin and in defining the basis for the determination at one minute:* The serums of thirty-eight consecutive patients with mild to severe degrees of jaundice were studied. In all cases the jaundice was due either to biliary obstruction or parenchymal liver disease. Direct reacting bilirubin determinations were made at one-half minute, one minute, and every minute thereafter for fifteen minutes, following which the total bilirubin values were measured. The determinations were expressed as percentages of the fifteen-minute reading, and color development curves were plotted. Samples of fistula bile, hemorrhagic plural fluid, and hemolytic jaundice serum were similarly studied and compared.

(e) *In studying the comparative incidence of abnormal values with the 1'B and TB tests in various liver diseases:* Records of 307 unselected cases from the Veterans and University hospitals were reviewed. The diagnosis in each case was believed to be unequivocal. Both sexes and all age groups were represented. However, the sample consisted predominantly of males in the older age groups. Included were 110 cases of hepatic cirrhosis; forty-nine cases of viral hepatitis; fifty-three cases of obstructive jaundice, including obstruction by neoplasm, stone, or stricture; nineteen cases with congestive heart failure; and seventy-six cases with approximately forty miscellaneous diseases including lymphomas, metastatic neoplasms, abdominal abscesses, pancreatitis, ulcerative colitis, malaria, empyema of gall bladder, and periarteritis nodosa. For each disease group, the incidence of abnormal scores and of various ranges of 1'B and TB scores was recorded as shown in Table VI. The limits of normal used were those found by the analyses of section (a).

(f) *In evaluating the discriminative value of the bilirubin ratio (1'/T × 100):* Records of 614 cases with various diseases affecting the liver, including the 307 cases noted above, were reviewed. Readily available records were used, but, in general, cases were selected consecutively starting with the most recent admissions and working backward. Of the total, 338 cases were observed in the Veterans Hospital and 276 in the University Hospitals. Four different laboratories and at least fifteen different technicians were concerned with the bilirubin determinations at one time or another. Included in the sample of 614 patients were 155 cases of cirrhosis; 110 cases of viral hepatitis; 164 cases of obstructive jaundice including stone, stricture, and neoplasm; forty cases of congestive heart failure; and 145 cases with approximately fifty miscellaneous diseases similar to those listed above. No attempt was made to classify these patients with respect to age or sex, but approximately two-thirds were males in the older age group.

Review was also made of records of thirty patients with hemolytic anemia. Each case was readily classified as such on the basis of the clinical features and the presence of reticulocytosis and (or) significant elevation of the fecal urobilinogen. Half were males, half females. Sixteen were of the congenital type, five idiopathic acquired, two acquired secondary to known diseases, and seven unclassified.

Bilirubin ratios were calculated for each of the six disease groups, and frequency distributions of these ratios were plotted. It was found that the distributions varied with the level of the total bilirubin, so the groups, with the exception of the forty congestive failure cases, were subdivided into three levels: TB 0 to 1.5 mg. per cent, TB 1.6 to 2.0 mg. per cent, and TB $>$ 2.0 mg. per cent for additional comparisons.

Laboratory Methods.—

The Malloy-Evelyn method[12] for determining serum bilirubin was used, modified to include a determination at one minute as suggested by Ducci and Watson.[13] The essential features of the procedure are as follows:

(a) 0.5 ml. of serum or plasma is added to 9.5 ml. of distilled water in a colorimeter tube, and after mixing, 5.0 ml. of diluted serum is transferred to each of two tubes. If the serum is quite icteric, 0.1 ml. of it and 9.9 ml. of distilled water should be used. If the 1:20 dilution has been used and the one-minute galvanometer reading is below 40.0, the determination should be repeated with the 1:100 dilution in order to be well within the range of accuracy of the Evelyn photoelectric colorimeter.

(b) 1.0 ml. of diazo blank solution is added to tube 1, and 1.0 ml. of diazo reagent to tube 2. Tube 2 is read at exactly one minute after the addition of the diazo reagent using the center setting obtained by tube 1 with filter 540 and 6 ml. aperture of the Evelyn colorimeter. This gives the prompt direct bilirubin (1′B) reading.

(c) 6.0 ml. of methyl alcohol are then added to each of the two tubes. Tube 2 is read again at the end of fifteen to thirty minutes using tube 1 as a blank again. This gives the total bilirubin (TB) reading.

Directions for preparing reagents and a permanent graph of standards are given in the paper of Malloy and Evelyn.[12] It may be noted that in the standard solutions they used there were variable small amounts of chloroform, which is known to accelerate the diazo reaction and increase its intensity. Careful study has shown, however, that within the range of chloroform used no variation is introduced.

Statistical Terms.—

All figures indicating variation about means (denoted by $\bar{x}$) are standard deviations, symbolized by s or SD. The coefficient of variation, CV, represents variation related to the mean, i.e., relative variation. The 5 per cent or 1 per cent points represent statements of probability, based either on the empirical distributions obtained or on appropriate distributions utilized in tests of significance, and indicate the proportion of all cases having scores exceeding the given points. The analysis of variance is a procedure for fractionating the total variation observed into components each of which may be evaluated by itself or in comparison with the others. The variance ratio or F value represents the ratio of two mean squares or variances and is the test of significance utilized in the analysis of variance. The terms correlation or regression refer to linear relationships unless otherwise specified. The correlation ratio, η, is a measure of nonlinear correlation whose interpretation is essentially similar to that of the product-moment correlation, r. χ^2 is used here as a test of independence in determining the significance of differences between per cents. The critical ratio, used here for testing the significance of differences between means, is the ratio of a given difference related to its standard error.

RESULTS

(a) *Normal Limits of the 1′B and TB Tests.*—The frequency and cumulative frequency distributions of these tests are plotted for the norm group in

Figs. 1 and 2. The distributions are positively skewed, that for the TB more so than for the 1'B. In addition to the mean and standard deviation, the upper 5 per cent and 1 per cent points of these empirical distributions were calculated, on the assumption that any future similar samples will have essentially similar distributions. At or below the 5 per cent and 1 per cent points fall 95 per cent and 99 per cent of the cases, respectively.

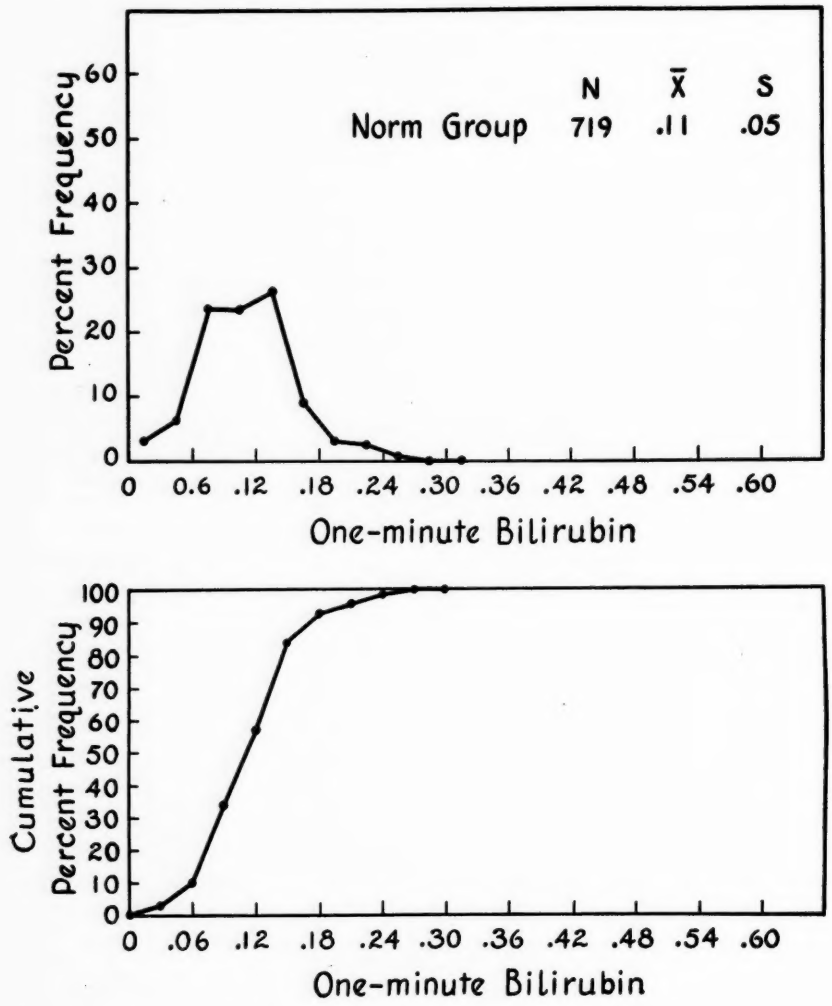

Fig. 1.—Frequency distributions of one-minute serum bilirubin concentrations (mg. per cent) in normal subjects. 5 per cent point 0.19 mg. per cent, 1 per cent point 0.24 mg. per cent.

The mean of the 1'B distribution is 0.11 ± 0.05 mg. per cent. The 5 per cent point is 0.19 mg. per cent, the 1 per cent point 0.24 mg. per cent. The mean of the TB distribution is 0.62 ± 0.25 mg. per cent. The 5 per cent point is 1.06 mg. per cent, the 1 per cent point 1.49 mg. per cent; or with sufficient accuracy 1.10 and 1.50 mg. per cent, respectively. The exact point selected to demarcate

the limits of normal for each of these distributions depends largely on how frequently one is willing to be wrong in calling a given value abnormal. Slight consideration is given also to mnemonic convenience. With small likelihood of error, the upper limit of normal for the 1'B determination may be taken as 0.25 mg. per cent, for the TB determination as 1.50 mg. per cent.

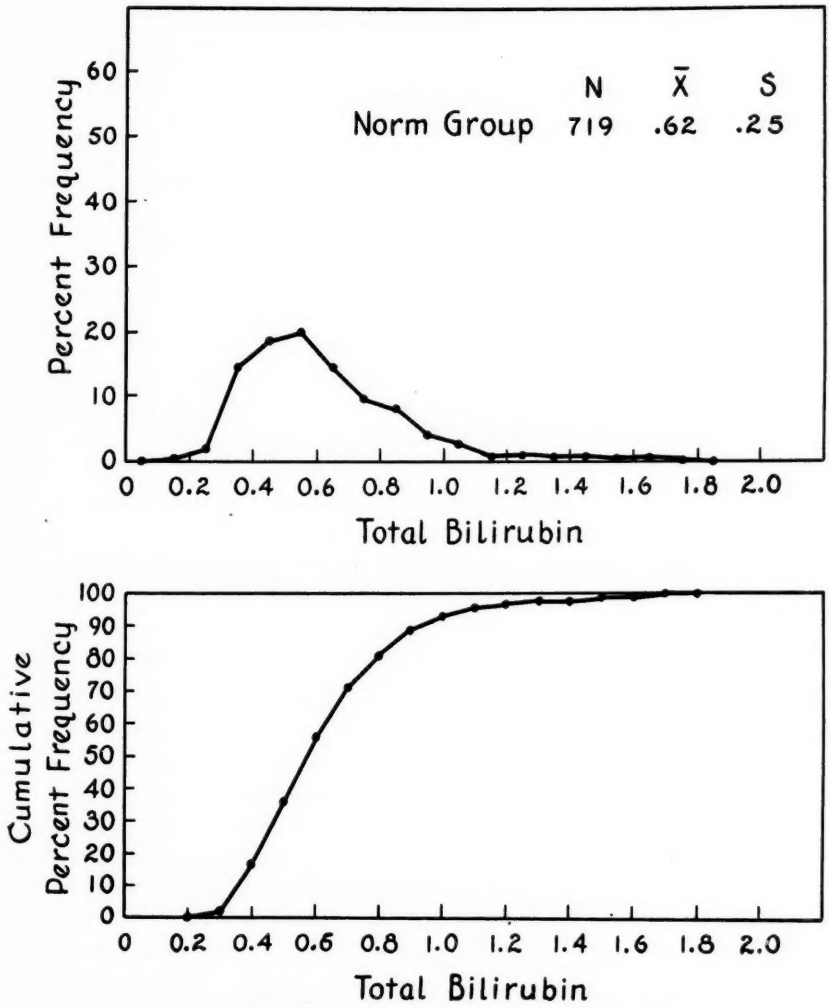
N
$\bar{X}$
S
Norm Group 719 .62 .25
Percent Frequency
0 10 20 30 40 50 60
0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
Total Bilirubin
Cumulative Percent Frequency
0 10 20 30 40 50 60 70 80 90 100
0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
Total Bilirubin

Fig. 2.—Frequency distributions of total serum bilirubin concentrations (mg. per cent) in normal subjects. 5 per cent point 1.10 mg. per cent, 1 per cent point 1.50 mg. per cent.

In view of the skewed nature of these distributions, the continuity of each tail with the body of the curve, and the presence within these regions of cases without histories of hepatitis or recurrent mild episodes of jaundice, it is unlikely that for this sample the higher values under the curves are to be accounted for by subjects having possible residuals of hepatitis or constitutional hepatic dysfunction. To set a lower limit of normal on this basis is to truncate the distributions arbitrarily.

(b) *Analyses of Variance. Test-Retest Reliability.*—The results of the analyses of variance are summarized in Tables I, II, III, and IV.

Results with the one-minute bilirubin test in normal individuals are summarized in Table I. The error mean square, which is a measure of the effect of chance variation, is 0.00067. Comparisons of all other effects with this allow us to test the significance of their contribution to the total variation observed. In this instance each main effect (contribution of each factor taken by itself) is highly significant. However, the interactions of individual with day-to-day and technical variations are also significant. Comparisons of the main effects with these significant interactions enable us to test the significance of the contribution of each variable uninfluenced by its association with another simultaneously operating variable. Thus comparison of the mean square for Da with that for the interaction Da × I gives a variance ratio of 4.51 instead of 12.09 for the effect of average day-to-day variation. Similarly comparison of Du with Du × I gives a variance ratio of 11.17 instead of 28.36 for the effect of average variations among duplicates. The average daily and duplicate variations are thus significant though far less so than was apparent at first. The scores obtained on individual subjects also varied significantly from day to day and determination to determination. The average absolute difference between values obtained on two successive days was 25.8 per cent of the larger value. One can therefore conclude that in addition to the significant variation among individuals, which one expects, physiologic and technical factors contribute significantly to the variability of this test in the normal range. The operation of these factors, together with the narrow range over which normal measurements are taken, results in the test's being very unreliable if any conclusion is drawn other than that a given value falls within the normal range. The test-retest correlation is only 0.26.

Results with the one-minute bilirubin test in abnormal patients are summarized in Table II. The error mean square is 0.0227, and the only main effect of significance is that due to average variation among individuals, which one expects to be great. There is also significant variation from day to day between scores of individual patients as evidenced by the significant Da × I interaction. The average absolute difference between the scores obtained on two successive days is 10.6 per cent. Technical factors do not contribute significantly to the over-all variation observed in these patients. In this range the test is highly reliable, the test-retest correlation being 0.996.

Similar analyses were made of the total bilirubin data. In Table III are listed the results of analyzing the variation in normal subjects. Average differences among duplicates contribute significantly to the total variation as evidenced by the significant variance ratio of 10.60. There is also significant individual variation in the scores obtained on successive days (Da × I interaction significant). The average absolute difference between the results obtained on two consecutive days is 16.1 per cent. However, the difference between the means of each day's determinations is not significant. Differences in technique thus contribute significantly to the total variation in this test; average daily differences do not. The test in normal individuals is reasonably reliable, the test-retest correlation being 0.89.

TABLE I. ANALYSIS OF VARIANCE OF ONE-MINUTE BILIRUBIN IN NORMAL SUBJECTS

SOURCE OF VARIATION	DF	SUM OF SQUARES	MEAN SQUARE	F
Da	1	0.0081	0.0081	12.09†
I	31	0.0972	0.0031	4.63†
Du	1	0.0190	0.0190	28.36†
Da × I	31	0.0571	0.0018	2.68†
Da × Du	1	0.0001	0.0001	0.15
I × Du	31	0.0534	0.0017	2.54†
Da × I × Du	31	0.0209	0.00067	
Total	127	0.2558		

Additional comparisons: Da/(Da × I), F = 4.51*
Du/(I × Du), F = 11.17†

*Significant (5 per cent, but not 1 per cent point).
†Highly significant (1 per cent point or better).

TABLE II. ANALYSIS OF VARIANCE OF ONE-MINUTE BILIRUBIN IN ABNORMAL SUBJECTS

SOURCE OF VARIATION	DF	SUM OF SQUARES	MEAN SQUARE	F
Da	1	0.0182	0.0182	0.80
I	30	3565.4629	118.8488	5235.63†
Du	1	0.0369	0.0369	1.63
Da × I	30	7.7141	0.2571	11.33†
Da × Du	1	0.0057	0.0057	0.25
I × Du	30	0.9775	0.0326	1.44
Da × I × Du	30	0.6811	0.0227	
	123	3574.8964		

†Highly significant.

Results of the corresponding analysis in abnormal patients are listed in Table IV. There is marked variation among individuals as expected. The initial daily and duplicate comparisons appear significant. When corrected for the individual differences between days (Da × I) and between duplicates (I × Du), respectively, these main effects are insignificant. The over-all variation among abnormal patients is thus almost completely due to average differences among the patients or to individual differences from day to day. The average absolute difference between scores of successive days is 7.1 per cent. The test is highly reliable in this range, the test-retest correlation being 0.996.

These analyses have quantitatively defined the major sources of variation of these tests. As was expected, variation among the individual subjects contributes the most to the over-all variation of the tests. This is particularly true among abnormal patients where a wide range of scores is possible. The variation of each individual's scores from day to day (physiologic variation) contributes significantly to the over-all variation in every instance, though the average daily variation is generally insignificant. Technical factors contribute significantly to the variation of both tests in the normal range, but are of no consequence in the abnormal range. Both tests are highly reliable in the abnormal range. The TB is reasonably reliable in the normal range also. The 1′B is unreliable within its narrow normal range for any purpose other than distinguishing a given score as normal.

(c) *Correlation of 1′B and TB in Regurgitation Jaundice.*—The scatter diagram relating the 1′B and TB is given in Fig. 3. There is no segregation of points according to any given disease group. However, there are relatively

TABLE III. ANALYSIS OF VARIANCE OF TOTAL BILIRUBIN IN NORMAL SUBJECTS

SOURCE OF VARIATION	DF	SUM OF SQUARES	MEAN SQUARE	F
Da	1	0.0094	0.0094	1.52
I	31	6.4130	0.2069	33.37†
Du	1	0.0657	0.0657	10.60†
Da × I	31	0.3681	0.0119	1.92*
Da × Du	1	0.0345	0.0345	5.57*
I × Du	31	0.3118	0.0101	1.63
Da × I × Du	31	0.1930	0.0062	
Total	127	7.3955		

*Significant.
†Highly significant.

TABLE IV. ANALYSIS OF VARIANCE OF TOTAL BILIRUBIN IN ABNORMAL SUBJECTS

SOURCE OF VARIATION	DF	SUM OF SQUARES	MEAN SQUARE	F
Da	1	2.0104	2.0104	44.28†
I	22	8799.3587	399.9709	8809.93†
Du	1	0.3913	0.3913	8.62†
Da × I	22	23.6596	1.0754	23.69†
Da × Du	1	0.0109	0.0109	0.24
I × Du	22	2.2487	0.1022	2.25*
Da × I × Du	22	0.9991	0.0454	
Total	91	8828.6787		

Additional comparisons: Da/(Da × I), F = 1.87, Insignificant
Du/(I × Du), F = 3.83, Insignificant

*Significant.
†Highly significant.

few hepatitis or obstructive jaundice cases with total bilirubin values below 2.0 mg. per cent. The scatter of points increases as the bilirubin values decrease below the one-minute bilirubin level of 1.0 mg. per cent. Within the normal range the scatter for patients with liver disease is the same as that for normal subjects. In the 1′B region of 0.45 to 1.0 mg. per cent the correlation, based on 71 cases, is 0.62. Above this point there are 349 cases for whom the correlation is 0.96, a nearly perfect relationship. The regression of total bilirubin (y) on one-minute bilirubin (x) in this region is $\log y = 0.9 \log x + .3061$ or $y = 2.02x^{0.9}$, very nearly a relation of two to one. There is no marked variation about this curve irrespective of the use of rectangular or logarithmic coordinates, and despite the performance of the tests in four completely independent laboratories. In view of this, the scatter apparent in an analogous correlation diagram presented by Klatskin and Drill[18] seems excessive. The lack of segregation among the various disease types in the correlation plot is consistent with the extensive overlapping found for the same groups when frequency distributions of the bilirubin ratios are compared, as is shown in section f below.

(d) *Variations in Color Development Curves of Direct Reacting Bilirubin.*—The variations in these curves are summarized in Table V in which are recorded the means, standard deviations, ranges, and coefficients of variation of the percentage readings at each time interval. Examination of a plot of the average points in Fig. 4 reveals a curve with a nearly vertical component (prompt reaction) covering the first half-minute, and a largely horizontal com-

ponent (slow or delayed reaction) covering the last 12 minutes. The slope of the curve changes rapidly in the region of one-half to three minutes, the greatest change occurring in the interval from one-half to two minutes. This interval is most simply represented for these data by the determination at one minute. The relative variation about these means is greatest initially and decreases rapidly. The coefficients of variation at one-half, one, two, and three minutes are, respectively, 15.8, 11.6, 10.5, and 8.1 per cent.

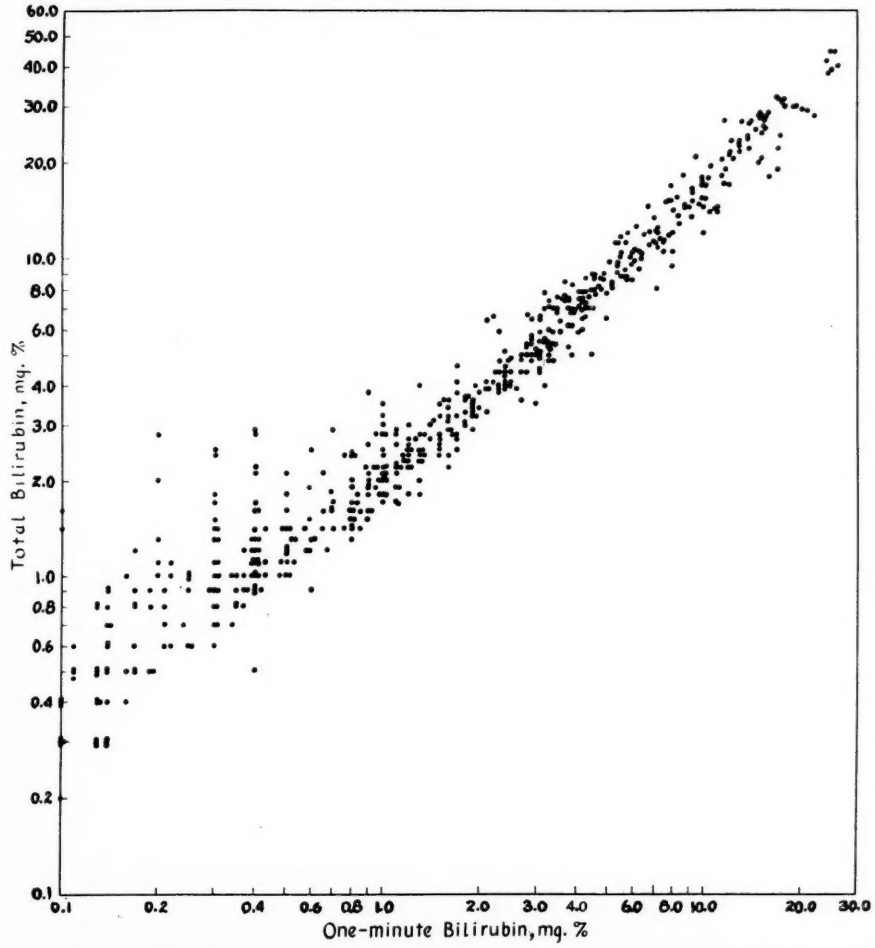

Fig. 3.—Relationship of total bilirubin and one-minute bilirubin in patients with parenchymal liver disease or biliary obstruction (logarithmic coordinates).

Examination of the color development curves for each individual reveals that the rate of change of the slope of every curve is maximal in the region of one-half to two minutes. Analysis of the data presented by Klatskin and Drill[18] in their Tables I and II reveals essentially the same phenomenon, though there were a few instances in which a later portion of the curve had a steplike form. This region can be represented in simplest fashion by the determination at one minute, though a measurement at one and one-half minutes or any intermediate

point in the interval would give essentially the same information. The one-minute value thus represents approximately the point of most rapid change from a prompt to a slow reaction and is therefore a useful quantitative measure of the prompt direct reacting serum bilirubin. This use of the one-minute bilirubin as a measure of the prompt direct reacting serum bilirubin is based on the actual characteristics of the color development curve and is independent of any theoretical interpretations of that curve.

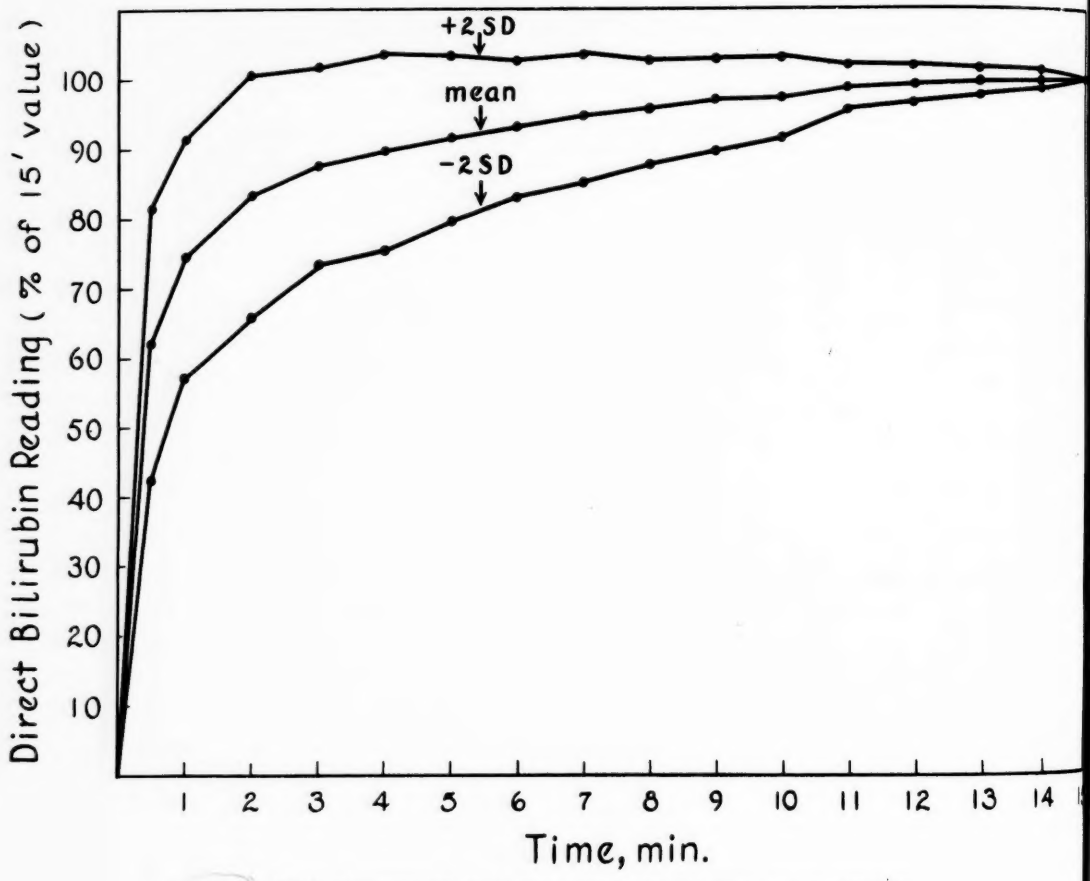

Fig. 4.—Color development time curve of direct reacting serum bilirubin.

The relative amount of prompt direct reacting bilirubin will naturally alter the exact form of such reaction curves. The extremes are well illustrated by curves of fistula bile and hemorrhagic pleural fluid, examples of which are given in Fig. 5, along with serums from cases of hemolytic jaundice, erythroblastosis fetalis, and obstructive jaundice. With fistula bile the reaction curve rose within a minute to its maximum value and there was no further rise with addition of alcohol, i.e., all of the bilirubin was prompt direct in type. With hemorrhagic (pleural) fluid there was no color development until alcohol was added, indicating that all of the bilirubin was indirect reacting. The hemolytic jaundice and obstructive jaundice serums show intermediate reactions.

(e) *Comparative Incidence of Abnormal Values with the 1'B and TB Tests in Various Liver Diseases.*—In Table VI are recorded the incidence of normal and abnormal scores as well as 1'B and TB scores within certain specified ranges for each disease group. In the lower six lines of the table the frequency of occurrence of values in three selected intervals are recorded for each test. Since the values recorded from the laboratory records were generally of the same order of magnitude as the maximal ones listed in the patients' charts, these figures

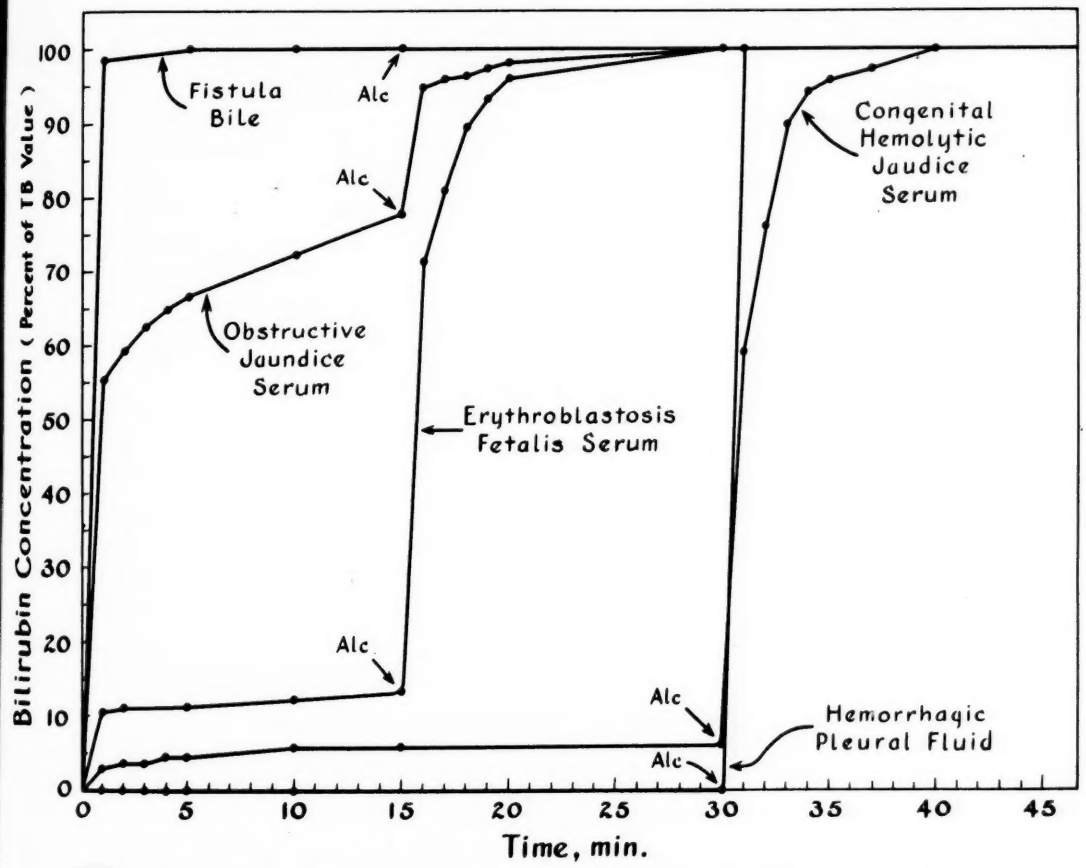

Fig. 5.—Color development time curves of direct and indirect reacting bilirubin in fistula bile, hemorrhagic pleural fluid, hemolytic jaundice serum, and obstructive jaundice serum.

portray approximately the comparative incidence of bilirubin scores at various levels, according to disease grouping. It is apparent that relatively few hospitalized cases of viral hepatitis or obstructive jaundice fail to have moderate or severe jaundice at the height of their illness. Over half the cirrhosis patients on the other hand have total bilirubin values falling within normal limits, and only one-third have moderate or severe degrees of jaundice. Forty-four per cent have one-minute values between 0.25 and 1.0 mg. per cent in contrast to 29 per cent with normal values and 27 per cent with values of 1.0 mg. per cent or

TABLE V. VARIATIONS IN THE DIRECT BILIRUBIN COLOR DEVELOPMENT CURVES (VALUES EXPRESSED AS PERCENTAGES OF FIFTEEN MINUTE READING)

TIME INTERVAL	MEAN	SD*	± 2 SD	RANGE	CV†
½	62.0	9.8	42.4- 81.6	32.0- 79.1	15.8
1	74.4	8.6	57.2- 91.6	42.0- 85.7	11.6
2	83.2	8.7	65.8-100.6	50.0- 93.8	10.5
3	87.6	7.1	73.4-101.8	59.0- 95.3	8.1
4	89.7	7.0	75.7-103.7	64.0- 97.1	7.8
5	91.6	5.9	79.8-103.4	69.0-100.0	6.4
6	92.9	4.9	83.1-102.7	76.0-100.0	5.3
7	94.3	4.6	85.1-103.5	81.0-100.0	4.9
8	95.3	3.7	87.9-102.7	84.2-100.0	3.9
9	96.4	3.3	89.8-103.0	84.2-100.0	3.4
10	97.2	2.9	91.4-103.0	87.7-100.0	3.0
11	98.7	1.6	95.5-101.9	93.0-100.0	1.6
12	99.1	1.4	96.3-101.9	93.0-100.0	1.4
13	99.5	0.9	97.7-101.3	96.5-100.0	0.9
14	99.7	0.7	98.3-101.1	96.5-100.0	0.7
15	100.0	0	—	—	0

*SD = Standard deviation.
†CV = Coefficient of variation.

greater. The relative incidence in this intermediate one-minute bilirubin range for patients with congestive failure and miscellaneous diseases associated with liver dysfunction is similar, being 58 per cent and 43 per cent, respectively.

The comparative over-all incidence of abnormal values with each test is listed in lines two and three of the table. Of the 110 patients with cirrhosis, 71 per cent had abnormal one-minute bilirubin scores and 43 per cent had abnormal total bilirubin scores, an incidence difference that is highly significant ($\chi^2 = 17.8$, $P < 0.001$). For the seventy-six cases with miscellaneous diseases the corresponding figures were: 1′B, 58 per cent abnormal; TB, 29 per cent abnormal; again a highly significant difference ($\chi^2 = 13.0$, $P < 0.001$). Similar comparisons for the hepatitis and obstructive jaundice groups are of little value since such a large proportion of these cases had severe degrees of jaundice. On the basis of individual observations one may anticipate that in the incipient stages of these diseases findings similar to those in the cirrhotic and miscellaneous disease groups will obtain. The congestive failure group had findings similar to those of the cirrhotic group; however, the number of cases was small and the difference observed could have occurred by chance ($\chi^2 = 1.73$, $P > 0.10$).

It is thus apparent that in those diseases in which relatively few cases have severe jaundice the one-minute bilirubin will indicate abnormality with greater frequency than the total bilirubin. This is even more apparent when the comparisons in lines four and five of the table are made. In no disease group are there more than one or two cases having normal one-minute bilirubin and abnormal total bilirubin scores. There were none in the important cirrhosis group. In contrast 28 per cent of this group had normal total bilirubin and abnormal one-minute bilirubin values. For the miscellaneous and congestive failure groups the corresponding figures were 32 per cent and 42 per cent, respectively. Results similar to these were observed by Cantarow, Wirts, and Hollander[19] for direct bilirubin determined at thirty minutes.

TABLE VI. COMPARATIVE INCIDENCE OF NORMAL AND ABNORMAL VALUES WITH THE ONE-MINUTE AND TOTAL BILIRUBIN TESTS, BY DISEASE TYPE*

CLASSIFICATION	CIRRHOSIS		VIRAL HEPATITIS		OBSTRUCTIVE JAUNDICE		MISCELLANEOUS		CONGESTIVE FAILURE	
	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%
Total cases	110	100	49	100	53	100	76	100	19	100
Abnormal 1′B	78	70.9	48	98.0	52	98.1	44	57.9	13	68.4
Abnormal TB	47	42.7	45	91.8	44	83.0	22	28.9	9	47.4
Normal 1′B, Abnormal TB	0	0	0	0	1	1.9	2	2.6	0	0
Abnormal 1′B, Normal TB	31	28.2	3	6.1	9	17.0	24	31.6	8	42.1
1′B $<$ 0.25 mg. %	32	29.1	1	2.0	1	1.9	32	42.1	6	31.6
0.25 $\leqq$ 1′B $<$ 1.00 mg. %	48	43.6	7	14.3	18	34.0	33	43.4	11	57.9
1′B $\geqq$ 1.00 mg. %	30	27.3	41	83.7	34	64.2	11	14.5	2	10.5
TB $<$ 1.5 mg. %	63	57.3	4	8.2	9	17.0	54	71.1	10	52.6
1.5 $\leqq$ TB $<$ 2.0 mg. %	9	8.2	2	4.1	9	17.0	8	10.5	6	31.6
TB $\geqq$ 2.0 mg. %	38	34.5	43	87.8	35	66.0	14	18.4	3	15.8

*For purposes of these calculations the exact 1 per cent points of the norm group frequency distributions were taken as the limits of normal.

There were sixty-three cirrhosis patients with normal total bilirubin scores. Of this number thirty-one, or 49 per cent, had abnormal one-minute bilirubin values. Thus half the patients with normal TB values have abnormal 1′B values. The findings are essentially the same if the 5 per cent points are taken as limits of normal instead of the 1 per cent points. Similarly, of fifty-four miscellaneous group patients with normal total bilirubin scores twenty-four, or 44 per cent, had abnormal one-minute bilirubin scores. The corresponding figures for the hepatitis, obstructive jaundice, and congestive failure groups were 75 per cent, 100 per cent, and 80 per cent; however, these were based on small numbers of cases.

In the presence of liver disease relative increases of 1′B are nearly always much more pronounced than those of TB. This disproportionate rise in the 1′B is of particular value when TB values fall in the range of 1.6 to 2.0 mg. per cent, as may be illustrated with the sample of cirrhosis patients. Eight per cent had TB values of 1.6 to 2.0 mg. per cent. The average increase in TB over the normal limit was 17 per cent (range 7 to 33 per cent). For the same patients the average increase in 1′B over the normal limit was 209 per cent (range 60 to 300 per cent).

Though the one-minute bilirubin is clearly much more efficient than the total bilirubin in distinguishing an individual with liver disease, the tests are equally efficient in distinguishing a group of abnormal patients from a group of normal subjects. This is illustrated by the following comparison of the cirrhosis group with a sample of 100 normal males. Tests of the significance of the difference between the means of the two samples were calculated for each bilirubin test. The critical ratio (i.e., difference between means expressed in standard units) for the one-minute bilirubin test was 6.59 and for the total bilirubin test was 6.97. There is thus little difference in the efficiency with which these two tests distinguish the two groups, on the average. This is consistent with the finding of a high correlation between the one-minute and total bilirubin determinations in the abnormal range. From these results it is evident that among patients with liver disease, total bilirubin values within the normal

range, when they occur, are generally higher than those of normal individuals. However, such values fall within the limits of normal and in terms of the individual case cannot be used for diagnostic purposes.

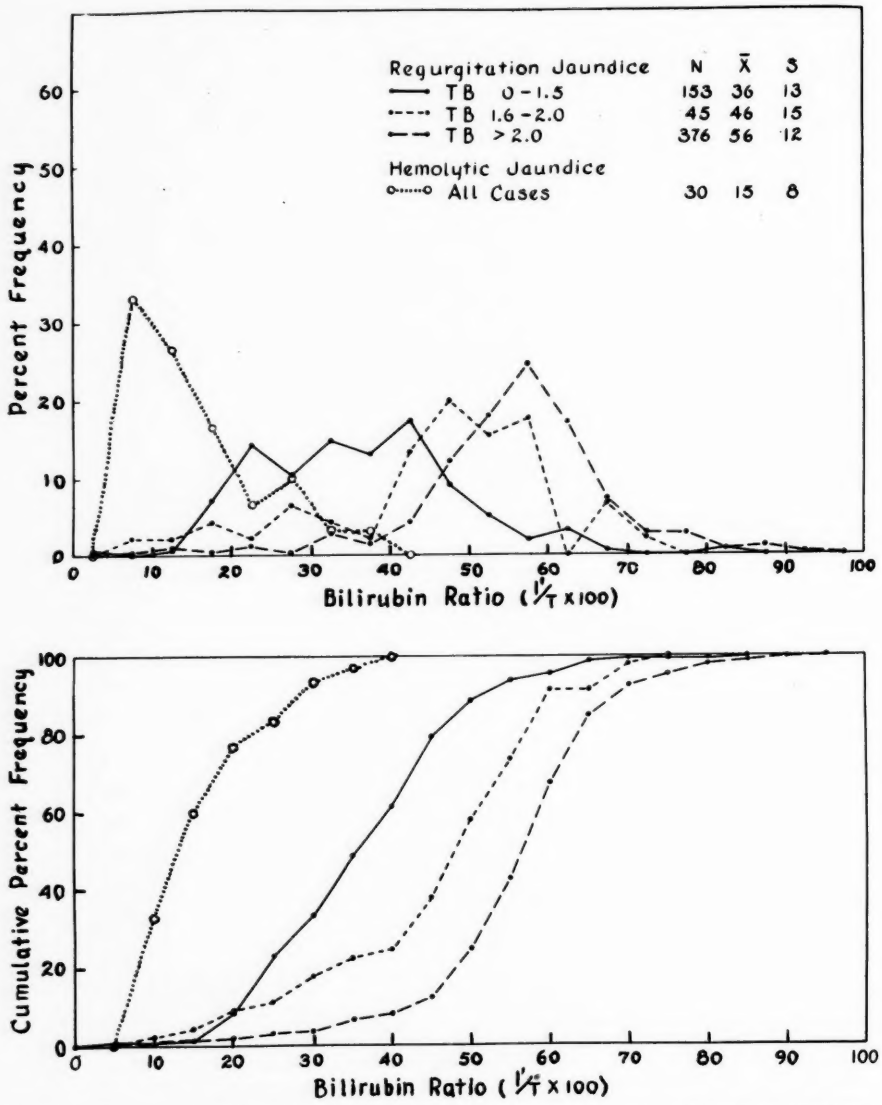
Regurgitation Jaundice N X̄ S
TB 0-1.5 153 36 13
TB 1.6-2.0 45 46 15
TB >2.0 376 56 12
Hemolytic Jaundice
All Cases 30 15 8
Percent Frequency
Bilirubin Ratio (1'/T x100)
Cumulative Percent Frequency
Bilirubin Ratio (1'/T x100)

Fig. 6.—Frequency distribution of the bilirubin ratio, by degree and type of jaundice.

(f) *Discriminative Value of the Bilirubin Ratio (1'/T × 100).*—Interpretation of the bilirubin ratio is dependent, within certain limits, on the degree of jaundice present. This is indicated in Fig. 6 by the frequency distributions of the bilirubin ratio for patients with regurgitation jaundice subdivided into the three levels: TB 0-1.5, TB 1.6-2.0, and TB > 2.0 mg. per cent. Corresponding to these three levels the mean bilirubin ratios are 36 ± 13 per cent,

46 ± 15 per cent, and 56 ± 12 per cent, respectively. The distribution for the sample of 30 hemolytic jaundice cases, included for contrast, has a mean value of 15 ± 8 per cent. The bilirubin ratio thus decreases significantly as the total bilirubin falls below 2.0 mg. per cent.

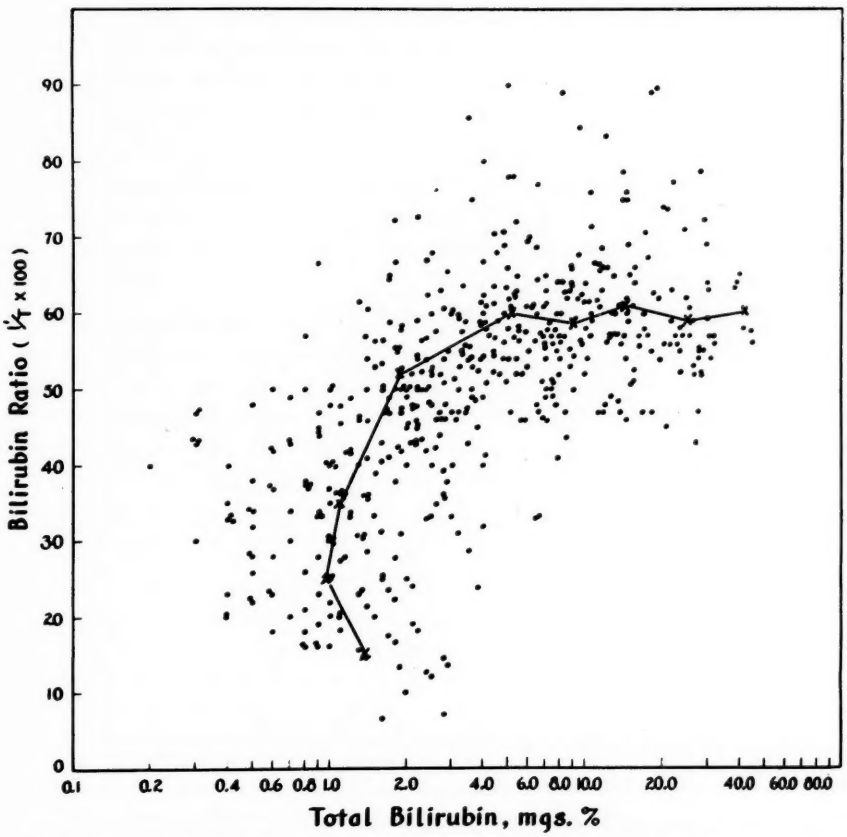

Fig. 7.—Relationship of bilirubin ratio and total bilirubin (semi-logarithmic coordinates).

The relationship of the bilirubin ratio to the degree of jaundice is more explicitly indicated in Fig. 7 in which the ratio is related to the logarithm of the total bilirubin. A moderately high curvilinear correlation is observed. The correlation ratio, η_{yx}, is 0.64. The curve drawn in the figure was fitted to the data by the method of averages and represents a simply calculated approximation to the curve of best fit. Consideration of the segment of the plot above the TB level of 2.0 or 3.0 mg. per cent reveals no appreciable relationship between the two variables in this region. The slight hook in the curve at its lower end is produced by a small proportion of cases having TB's of approximately 2.0 mg. per cent and low bilirubin ratios. All of the latter had cirrhosis.

Frequency distributions for the various disease groups are plotted in Fig. 8 for those cases with TB $>$ 2.0 mg. per cent, the only grouping containing sufficient numbers of cases for such comparisons. The average ratios

for the regurgitation-type cases vary from 51 per cent to 57 per cent. The over-all average for all such cases is 56 ± 12 per cent, being approximately the ratio anticipated from the equation, $TB = 2.02(1'B)^{0.9}$, previously calculated. The various groups overlap nearly completely, though the cirrhosis and congestive failure patients have lower mean ratios as a consequence of a greater proportion of cases falling below 40 per cent. The hemolytic cases show practically no overlap with any of the other groups. The average for the hemolytic group is 15 per cent, and 96 per cent of the cases have values less than 30 per cent. Of the regurgitation-type cases, the patients with cirrhosis had the lowest mean bilirubin ratio, 51 per cent. Only 9 per cent of these cases had ratios below 30 per cent. The well-known fact that hemolytic jaundice patients have very little prompt direct reacting bilirubin is reemphasized by this comparison, and the value of the bilirubin ratio in differentiating retention from regurgitation jaundice is self-evident.

One cannot differentiate among individuals with regurgitation jaundice by means of the bilirubin ratio. However, certain average comparisons reveal significant differences. The mean ratio of 51 per cent for the cirrhotic group is significantly less than the mean of 57 per cent for the hepatitis group (critical ratio = 2.95). A similar comparison is found with the obstructive jaundice sample. A significantly ($\chi^2 = 15$, $P < 0.001$) greater number of cirrhosis patients also have ratios below 40 per cent. Thus 21 per cent of the cirrhosis cases, 2 per cent of the hepatitis cases, and 4 per cent of the obstructive jaundice cases have ratios falling below this point.

The larger proportion of cirrhosis as well as miscellaneous group patients with ratios below 40 per cent is probably a consequence of including some patients having a hemolytic component or relatively greater hepatocellular than cholangiolar impairment. An illustrative example is the following brief summary of a case of cirrhosis with macrocytic hemolytic anemia.

Patient A. M.—Male, aged 54 years. Chronic alcoholism; jaundice, ascites, spider nevi; recurrent somnolence, coma, and death. Macrocytic anemia, hematinemia, reticulocytes 15 to 20 per cent. Fecal urobilinogen 950 mg. per day; urine urobilinogen 28 to 35 mg. per day; 1'B 5.4 mg. per cent; TB 20.5 mg. per cent; bilirubin ratio 26.3 per cent.

One would anticipate that the frequency distribution of bilirubin ratios for a group of such patients would fall somewhere between the distributions for the hemolytic and the regurgitation jaundice groups observed. The points causing the hook at the lower end of the curve of Fig. 7 probably represent patients of this type.

DISCUSSION

The results of the present study fully confirm previous observations as to the validity of quantitative measurement of the prompt direct or one-minute bilirubin, and show clearly how the value of the van den Bergh reaction in the clinical study of liver or biliary tract disease is enhanced by its use. The quantitative analyses presented clarify various apparently contradictory observations.

In the light of the results obtained, it is desirable to discuss more specifically the objections which have been raised as to the validity of the prompt reacting or one-minute serum bilirubin. The observation of Gray and Whidborne that the diazo color reaction does not obey Beer's law above concentrations of 1.6 mg. of bilirubin per 100 ml. of final solution is applicable to visual methods of measuring direct color development only, though the authors leave the im-

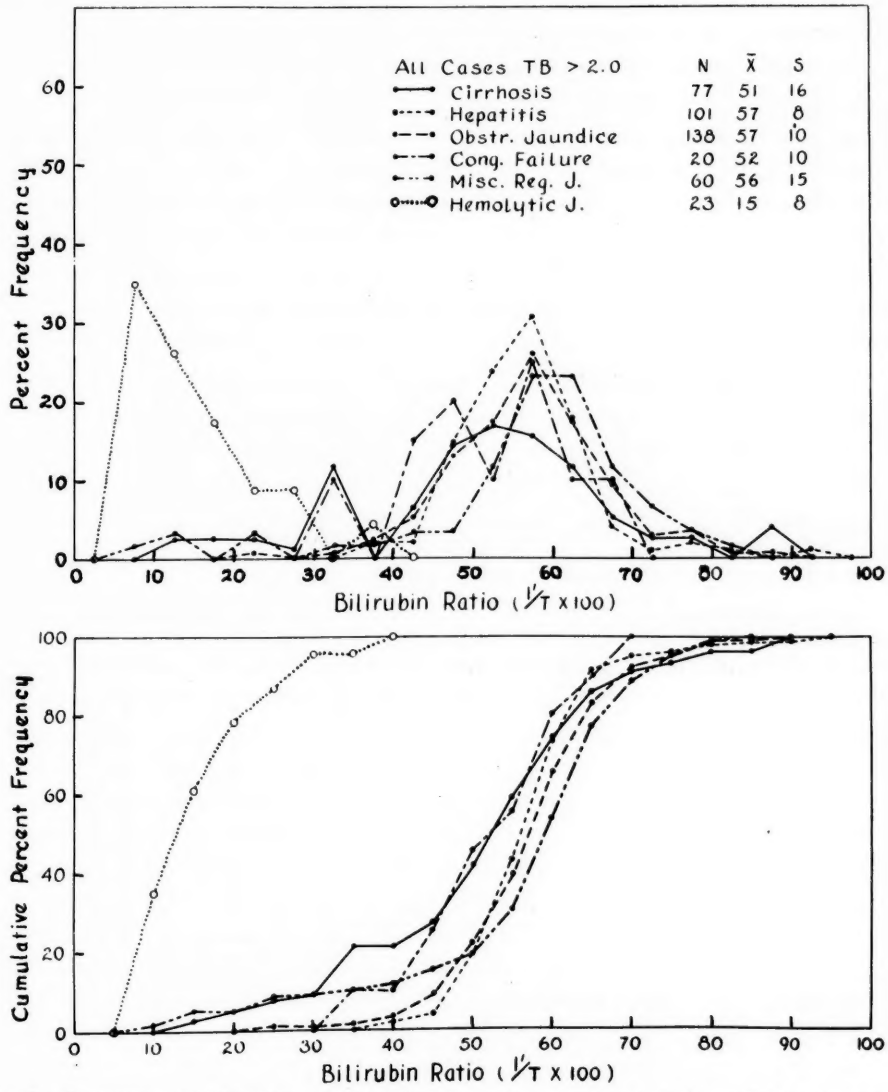

Fig. 8.—Frequency distributions of the bilirubin ratio in patients with moderate or marked jaundice, by disease type.

pression that it accounts for the results with the quantitative method previously reported by Ducci and Watson. In other respects the method used by Gray and Whidborne was not comparable to ours, particularly in the use of caffeine sodium benzoate which has a pronounced catalytic effect, very similar to that of alcohol itself.

The objection[18] that the time reaction curve of the direct van den Bergh is a variable diphasic curve representative of but a single rather than two differing components of bilirubin, deserves careful consideration. From a physical-chemical standpoint the character of the curve is consistent with the presence of two components with different speeds of reaction. This, of course, does not necessarily mean the presence of two bilirubins of differing chemical structure. It could equally well be due to two bilirubins existing in a different physical state, perhaps with respect to another molecule such as a protein. In essence this is the concept of Coolidge,[20] and more recently of Deenstra.[21] The hypothesis that the indirect reacting bilirubin is still attached to the original globin of the hemoglobin molecule while the prompt reacting type is a sodium bilirubinate adsorbed on the serum albumin is compatible with these views and with the interpretation that the time reaction curve is a true diphasic curve indicative of two components. It is recognized, however, that other possibilities have not been excluded. In this connection it is worth noting that a small fraction of the serum bilirubin has been found by Martin[22] to be tightly bound to α globulins.

Considerable emphasis has been given by others[18] to the observation of the same type of time reaction curve for a solution of crystalline bilirubin in a 1:8:1 mixture of chloroform, methyl alcohol, and water. This does not take into account, however, previous recognition that chloroform greatly accelerates the van den Bergh reaction. Hunter[23] showed years ago that pure bilirubin suspended in alcohol does not react with the diazo compound but that the addition of as little as 1 per cent of chloroform quickly brings about formation of the azo dye.

Objections to the clinical application of the bilirubin ratio to the ordinary case in the differential diagnosis of regurgitation jaundice seem justified. Its inadequacy in the distinction between medical and surgical jaundice was indicated in an earlier paper,[24] and the present study reveals clearly that a high correlation exists between the 1′B and TB in cases with appreciable regurgitation jaundice. There are, however, certain significant group differences which should not be ignored. These are found both in the means and at the extremes of the distributions. It should be noted also that the excellent correlation of 1′B and TB for the entire group of regurgitation jaundice cases is not in disagreement with the concept[9] of cholangiolar regurgitation of 1′B, as contrasted with hepatocellular retention of delayed or indirect reacting bilirubin. The relative influence of one or the other of these two factors might well be responsible for the scatter of values seen in Fig. 8.

It is desirable finally to comment on the relationship of the serum bilirubin to the occurrence of bilirubinuria. In the older literature it was often stated that the renal threshold for bilirubin was 2.0 mg. per cent. The study of hepatitis cases made it apparent that the threshold is quite variable not only from case to case but at different stages of the disease. Bilirubinuria was often noted in the preicteric or prodromal stage, or in cases of hepatitis without

jaundice when the total bilirubin was within normal limits.[9] In the convalescent stage, bilirubinuria disappeared as a rule when the one-minute bilirubin was between 0.8-1.2 mg. per cent, but occasionally much higher than this.

Bilirubinuria is not dependent on the total bilirubin concentration. Nor is it correlated with any given level of the one-minute bilirubin. Nevertheless, bilirubinuria has been observed only when the prompt reacting bilirubin is increased disproportionately, as indicated by the bilirubin ratios in Table VII, and its appearance is better correlated with such increases than with elevation of the total bilirubin per se. This, after all, does nothing more than reemphasize the correlation of "acholuric" jaundice with the finding of a delayed or indirect type of van den Bergh reaction. "Acholuric" jaundice is usually synonymous with retention jaundice, which includes hemolytic jaundice, constitutional hepatic dysfunction (familial nonhemolytic jaundice) and certain cases of isolated hepatocellular injury.

TABLE VII. CASES ILLUSTRATING RELATIONSHIP OF BILIRUBINURIA TO THE ONE-MINUTE AND TOTAL BILIRUBIN VALUES AND TO THE BILIRUBIN RATIO

CASE	DIAGNOSIS	SERUM BILIRUBIN		BILIRUBIN RATIO (1′/T × 100)	URINE BILIRUBIN
		1′B	TB		
1	Cirrhosis	0.5	0.7	71.4	0
2	Hepatic Carcinomatosis	0.5	1.0	50.0	+
3	Hepatitis (s jaundice)	0.8	1.8	44.4	+
4	Hepatitis (subsiding)	1.2	2.6	46.2	+
5	Familial Hem. Jaund.	0.2	3.2	7.5	0
6	Familial Hem. Jaund.	0.3	3.5	8.6	0
7	Cirrhosis	1.9	4.0	47.5	+
8	Familial Hem. Jaund.	0.4	5.2	7.3	0
9	Common Duct Stone	6.4	8.4	76.2	+
10	Ca of Pancreas	6.1	8.7	70.1	+
11	Constitutional Hep. Dysf.	1.0	8.8	10.9	0
12	Familial Hem. Jaund.	0.7	9.8	7.2	0
13	F. H. J. and C. D. Stone	5.2	11.0	47.3	+
14	Cirrhosis	5.9	11.4	51.8	+
15	Acute Atrophy	5.2	14.7	35.4	0
16	Infantile Cirrhosis (?)	0.6	14.8	4.0	0
17	Common Duct Stone	20.0	29.4	68.0	+
18	Ca of Pancreas	17.8	31.8	56.0	+
19	Ca of Pancreas	27.0	44.5	60.7	+
20	Acute Atrophy	31.9	51.2	62.3	+

SUMMARY

The studies of this paper have defined quantitatively normal and abnormal variations of the one-minute and total serum bilirubin tests, and have evaluated the clinical significance of the one-minute bilirubin determination.

The mean normal one-minute bilirubin is 0.11 ± 0.05 mg. per cent. The mean normal total bilirubin is 0.62 ± 0.25 mg. per cent. The upper limit of normal for the one-minute determination is 0.25 mg. per cent; for the total determination, 1.5 mg. per cent.

Analyses of the variation of each test in normal and abnormal subjects emphasize the significant contribution of physiological factors to the over-all variation in every instance. Technical factors contribute significantly to the

variation of both tests in the normal range but are of no consequence in the abnormal range. Both tests are highly reliable in the abnormal range. The total bilirubin is also quite reliable in the normal range. However, the one-minute bilirubin is unreliable in the normal range for any purpose other than distinguishing a given score as normal.

In regurgitation jaundice the one-minute and total bilirubin scores are nearly perfectly correlated above a one-minute level of 1.0 mg. per cent. The equation relating the two variables in this region is $(TB) = 2.02\ (1'B)^{0.9}$, very nearly a two to one relationship. There is no segregation of points in the scatter diagram according to disease groups. Below this level the correlation decreases progressively.

Color development time curves of direct reacting bilirubin consist generally of two components, one prompt, the other slow or delayed. The rate of change of the slope of the curve is maximal in the neighborhood of one minute. The one-minute determination is therefore a useful quantitative measure of the amount of prompt direct reacting bilirubin.

The one-minute bilirubin is more effective than the total bilirubin in detecting an individual with liver or biliary tract disease, though the tests are equally efficient in differentiating a normal from an abnormal group of patients, on the average.

There is a significant curvilinear correlation ($\eta = 0.64$) between the bilirubin ratio ($1'/T \times 100$) and the total bilirubin. The bilirubin ratio decreases rapidly as the total bilirubin decreases below 2.0 mg. per cent. Above this level there is no appreciable correlation between the two.

There is nearly complete separation between the bilirubin ratio frequency distributions of patients with hemolytic jaundice and patients with regurgitation jaundice. The mean ratio for all hemolytic jaundice patients with $TB >$ 2.0 mg. per cent is 15 ± 8 per cent, and for the corresponding regurgitation jaundice patients 56 ± 12 per cent.

One cannot differentiate among individuals with regurgitation jaundice by means of the bilirubin ratio because there is relatively great overlapping of the frequency distributions for the various disease groups. Certain group comparisons, however, reveal significant differences in the means and at the extremes of the distributions.

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LABORATORY METHODS

A METHOD FOR THE DETERMINATION OF SMALL DOSES OF I-131 IN THE URINE

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THE use of I-131 has become increasingly important in the diagnosis of thyroid disease. The method of study has been primarily through urine excretion and thyroid uptake studies. Prior to this time tracer doses of I-131 have ranged from 5 to 100 microcuries in urinary excretion studies. Allen[1] has mentioned the feasibility of using one microcurie doses, using the Geiger-Mueller tube. The ability to use small doses of I-131 has made this procedure comparable to the routine roentgenogram of the chest as far as radiation hazard is concerned.

Since the introduction of the scintillation counter[2] employing the use of an innocuous dose of one microcurie of I-131, it has become desirable to devise a simple method for doing urinary excretion studies. This has been accomplished using the identical scintillation tube and equipment used in performing thyroid uptake measurements.

METHODS AND RESULTS

Subsequent to the administration of the tracer dose two specimens of urine, representing zero to six hour and six to twenty-four hour periods, are collected and measured. Fluid intake should be restricted to 1,000 ml. daily so that the total urine volume does not exceed 1,000 ml. per twenty-four hours when a one or two microcurie dose is used. In order to determine the amount of I-131 in the specimen, the total volume is shaken well and an aliquot of 250 ml. of urine is put into a pint size cardboard container. This is placed in the lucite holder on the top of the scintillation tube as represented in Fig. 1. Then the number of counts of this volume is recorded. The calculations are made as follows:

U = counts per second of patient's urine sample (250 ml.). S = counts per second of 1 μc. of I-131 in 250 ml. urine. $V = \frac{\text{volume of urine in ml.}}{250 \text{ ml.}}$

Microcuries present in total urine specimen equal $\frac{U}{S} \times V$. The method presented indicates that the scintillation counter can be used to determine urinary excretion of I-131, using an administered dose as small as one microcurie. With this method a high counting rate is obtained in hypothyroid states, whereas a low counting rate is to be expected in hyperthyroidism. We are presenting data obtained from thirty-six studies on thirty-five

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TABLE I. COMPARISON OF 24 HOUR URINARY I-131 EXCRETION VALUES IN VARIOUS THYROID STATES

SOURCE	CARRIER IODINE (μG)	DOSE I-131 USED (μC)	HYPERTHYROID			EUTHYROID			HYPOTHYROID			METHOD OF COUNTING
			NO. CASES	% MEAN	% RANGE	NO. CASES	% MEAN	% RANGE	NO. CASES	% MEAN	% RANGE	
McArthur et al.[3] (1948)	2-160	100	22	25	7-45	30	60	23-98				G-M; beta
Werner et al.[4] (1948)	Carrier free	50-75	28	17	6-27	30	52	28-76	5	55	48-62	
Oshry & Schmidt[5] (1948)			34		5-43	23		42-80	10		48-93	G-M; gamma
Keating et al.[6] (1947)	100	100	16	25	4-65	14	57	47-67				G-M; beta
Karr & Hursh[7] (1950)	Carrier free	50-100	32	29	5-52	8	61	38-71				G-M; beta
Mason & Oliver[8] (1949)	45	10	22		5-26	16		28-72	7		"Within normal range"	G-M; gamma
Goodwin & Harris (1951)	Carrier free	1	11	21	8-54	35	59	43-70	13	68	46-89	Scintillation; gamma

euthyroid controls, thirteen studies on eleven hyperthyroid patients, and fifteen studies on thirteen hypothyroid patients. As seen in Table I, the per cent I-131 excreted in the urine ranged from 8 to 54 per cent, with a mean of 21 per cent in the hyperthyroid group; the range varied from 43 to 70 per cent with a mean of 59 per cent in the euthyroid group; in the hypothyroid group the range varied from 46 to 89 per cent with a mean of 68 per cent. The results are comparable to those of previously described methods, tabulated in Table I.

In our method, gamma radiation is measured with the scintillation counter. This method eliminates absorption, pipetting, and drying errors common to other counting procedures. The use of glassware other than the collecting bottle has been dispensed with. Because of the simplicity of the procedure, it leaves only two sources of error, the collection of the sample and the counting accuracy of the instrument.

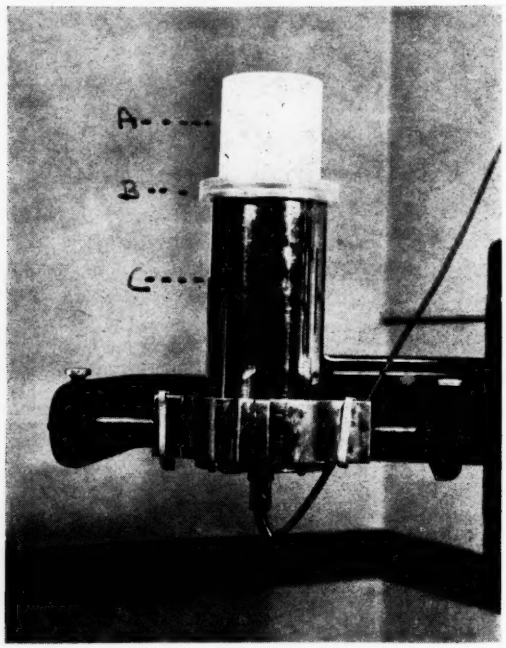

Fig. 1.—*A*, Cardboard container; *B*, lucite holder; *C*, scintillation tube.

CONCLUSIONS

A new method for measuring small doses of I-131 utilizing gamma counting is described.

The procedure is simple and useful in clinical medicine.

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THE BIURET REACTION FOR THE DETERMINATION OF PROTEINS—AN IMPROVED REAGENT AND ITS APPLICATION

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A RAPID and reliable method for determination of protein concentrations in small volumes of biological fluids is essential to many studies. Of chemical methods available, those based on the formation of the biuret complex stand out as most adaptable to this purpose. This paper is concerned with a modification of the biuret reagent distinguished by simplicity of use and preparation.

INSTRUMENTS AND MATERIALS

Optical densities were determined using a Beckmann spectrophotometer (Model DUV) and ten millimeter Corex glass cells. These cells are not seriously attacked by the reagents used.

As test proteins canine plasma fraction IV-6,[4] human plasma fractions IV-6, and II + III,[5] crystalline bovine plasma albumin,[2] and fresh human blood plasma were used.

Composition of Reagent and Standard Analytical Procedure.—On the basis of studies detailed below the reagent and procedure adopted for routine use in this laboratory are as follows: *Reagent:* 0.175 Gm. of $CuSO_4 \cdot 5H_2O$ dissolved in a few milliliters of water are placed in a 100 ml. volumetric flask, to which a mixture of 30 ml. of concentrated NH_4OH, 50 ml. of cold distilled water solution, and 20 ml. of saturated NaOH solution are added. The mixture, when brought to room temperature and made up to volume, is ready for use. The reagent as thus prepared is stable for several months at room temperature.

Procedure.—2.0 ml. of this reagent are added to 3.0 ml. of a protein solution containing between 0.05 and 1.25 Gm. protein per liter. The solutions are mixed, and optical density at 540 μ is determined immediately or up to ten minutes later. Protein concentration is determined using a calibration curve, such as that shown in Fig. 1.

RESULTS

Absorption Spectra.—Fig. 2 shows absorption curves for the reagent (maximum at 545 mμ) and for the reaction mixture containing a considerable excess of protein so that virtually all of the Cu ++ is bound as biuret complex. Optimal wave length for accurate determination of the biuret complex in the presence of reagent would be between 540 and 530 mμ. The former value was chosen for this procedure because many commercially available colorimeters are equipped with narrow band filters transmitting this wave length.

VARIATIONS IN RECENT COMPOSITION

a. *Copper content:* Fig. 3 shows the optical densities of reaction mixtures containing varying amounts of copper, as a function of protein content. The

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range of linear optical-density-to-protein-content relations is determined by the copper content in such fashion that at the upper limit of linearity approximately 3.3 M. of amino acid residues are present per 1.0 M. of Cu ++. The protein free "blank" reading increases with increasing copper content, rendering the determination of small protein concentrations unsatisfactory. The reagent composition given above was adopted as a compromise between these two limiting factors to yield a satisfactory range of application.

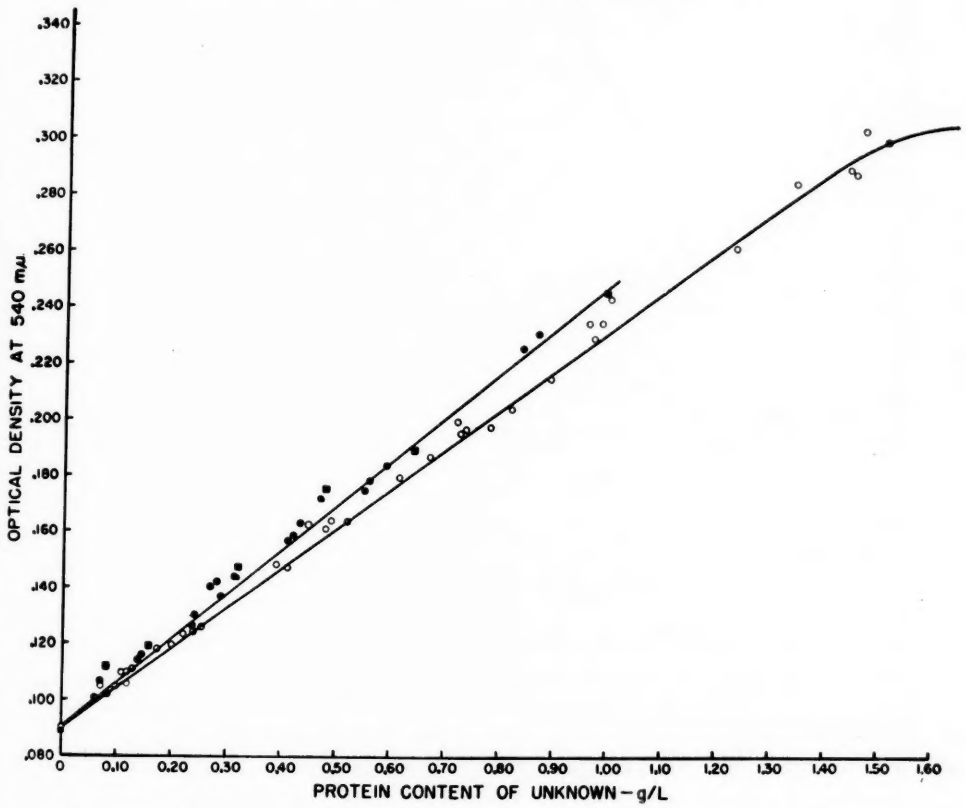

Fig. 1.—Relation between optical density at 540 μ of biuret reaction mixtures and protein concentrations at 23° to 28° C.

○ Fresh Human Plasma
■ Bovine Albumin
● { Human IV-6-4, Canine IV-6, Human II + III

b. *Ammonium hydroxide* in concentrations of 0.38 M. or greater is needed to maintain the cupric ion in solution. With a given protein concentration the optical density at 540 mμ of reaction mixtures decreases by only about 6 per cent as the initial NH_4OH concentration in the reagent is raised from 0.38 to 7.5 M. The 2.4 M. NH_4OH chosen for the reagent is convenient but noncritical.

c. *Sodium hydroxide:* The cuprammonium fails to react with proteins even in the presence of a large excess of NH_4OH. However, the addition of

NaOH in concentrations of 7.5×10^{-3} M. or greater results in prompt, quantitative formation of the biuret complex. Optical densities of such reaction mixtures are independent of NaOH concentrations between 7.5×10^{-3} and 4.0 M.; 1.9 M. or 0.19 M. NaOH concentrations in the reaction mixture are about equally suitable, the former being more convenient in practice because the reaction is completed almost immediately.

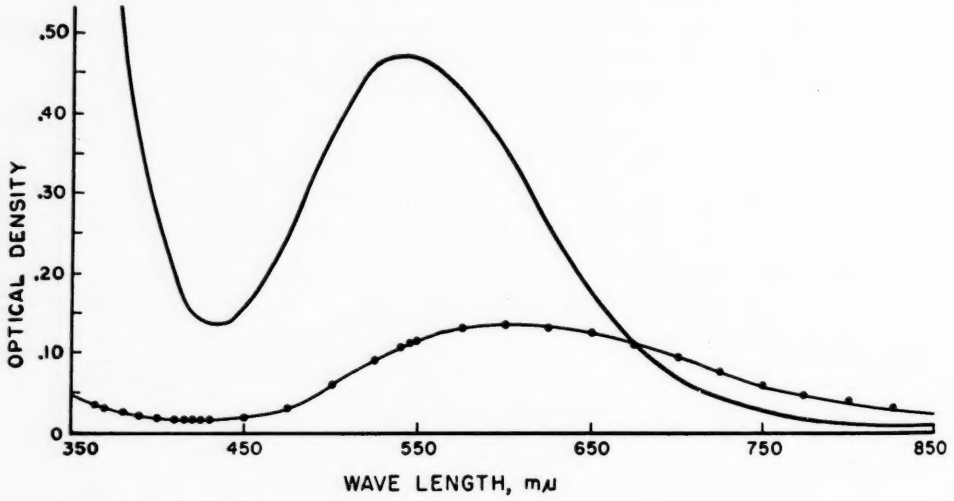

Fig. 2.—Absorption curves at 28° C., 10 mm. cells.
●——● Biuret reagent (2 ml. Biuret reagent plus 3 ml. distilled water).
——— Reaction mixtures (2 ml. reagent plus 3 ml. of 4.0g per liter of crystalline bovine plasma albumin).

d. *Turbidity and stability of biuret color:* Approximately thirty minutes after mixing reagent and protein solutions, the reaction mixture may begin to become cloudy, and a flocculent precipitate is formed. This is especially pronounced with tissue extracts, and is slight or absent with purified plasma albumin. Fig. 4 shows the changes in optical density as a function of time resulting in the case of blood plasma from the gradual formation of precipitate, and the return to initial optical density on removal of the precipitate by centrifugation after twenty-four hours. Longer periods of storage, up to four days, were tested using three samples of human plasmas, and three solutions of bovine plasma albumin. Optical densities after removal of the precipitate were found stable under these conditions.

Not only the biuret reagent, but also the copper-free NH_4OH-NaOH solution will result in the development of turbidity and floccules in plasma protein solutions.

The precipitate has a specific gravity very slightly below that of the reaction mixture, and is present in minute amounts only. A sample of this material, concentrated by centrifugation, was found to be largely extractable with ether, from which it could be precipitated with acetone-Mg Cl_2. This

precipitate was found to contain phosphorus. While further isolation and purification of this precipitated material was not attempted, odor, the solubility behavior outlined, and the presence of phosphorus suggest that one is dealing with lipids, largely phospholipids, protein bound in the native plasma, and released by the reagent.

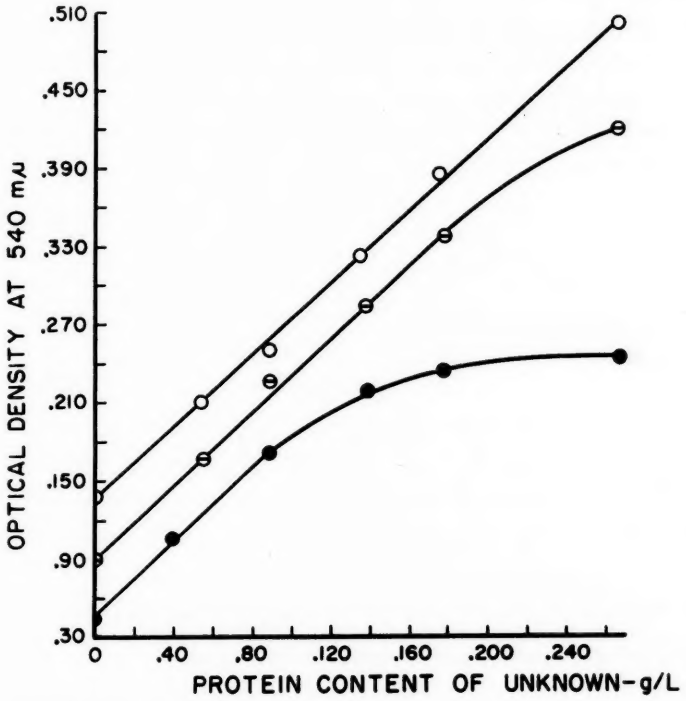

Fig. 3.—Optical density of reaction mixtures of different Cu content as a function of protein composition.

○——○ 6.0 × 10^{-4} M Cu++
⊖——⊖ 4.1 × 10^{-4} M Cu++
●——● 2.4 × 10^{-4} M Cu++

APPLICATION OF STANDARD PROCEDURES

Stock protein solutions (human blood plasmas, as well as solutions of the various plasma protein fractions) were standardized by determining their total nitrogen contents (duplicate semimicro Kjeldahl determinations, agreeing to within ±1 per cent in each case) and nonprotein nitrogen. For convenience only in the following discussion these protein nitrogen values were converted to "total protein" values by multiplying with a mean conversion factor of 6.25.

Plasma Proteins.—Blood plasmas from six patients were used to prepare solutions containing 0.06 to 1.4 Gm. per liter of total protein. These were mixed with biuret reagent and optical densities determined by the procedure described. Agreement between duplicate determinations was better than ±5 per cent in all cases, and within ±2 per cent in all samples between 0.20 and

1.00 Gm. per liter. These data are plotted against total protein content in Fig. 1. The effective extinction coefficient for the biuret complex formed from whole human blood plasma, as calculated from the blank and optical densities observed in the range of greatest accuracy (0.50 to 1.00 Gm. per liter, twelve determinations) is $T/E\,{}^{1\%}_{1\,cm.}$ (540 mμ) = 2.37 ± 0.04. This value also applies to rat and dog whole plasma.

On the other hand, using plasma fractions obtained by low temperature solvent fractionation a significantly higher extinction coefficient was obtained, $T/E\,{}^{1\%}_{1\,cm.}$ (540 mμ) = 2.82 ± 0.05 (cf. line 2, Fig. 1). There were no detectable differences between the (two) albumin preparations on the one hand, and the various globulins on the other.

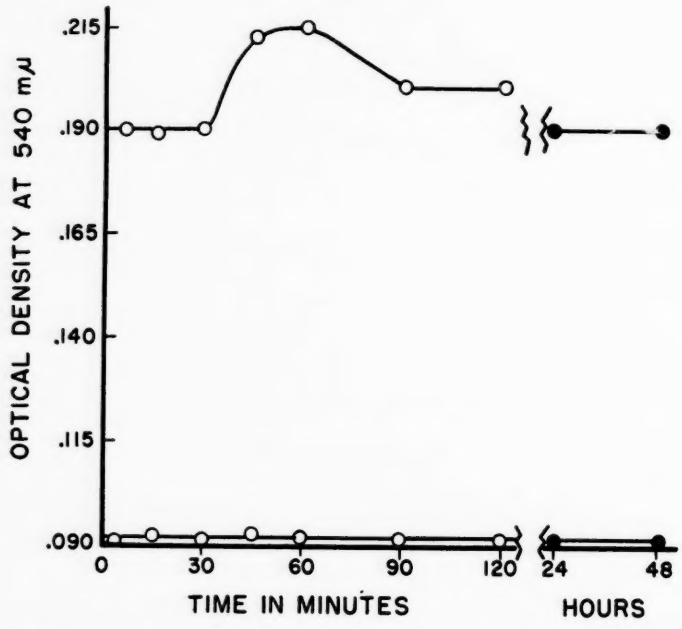

Fig. 4.—Changes in optical density of a reaction mixture containing 500 mg per liter of total protein (fresh canine blood plasma) as a function of time, compared with 2 ml. of reagent plus 3 ml. of distilled water read at the same time intervals.

Mixtures of purified human plasma albumin solutions and of whole plasma showed the predicted intermediate extinction coefficients calculated on the assumption that each protein in the mixture retained the extinction coefficient it had shown separately.

Whole plasma protein was precipitated under conditions comparable to those for the preparation of fraction V (40 per cent ethanol, T/2 = 0.11, pH = 4.80, temperature − 5° C.)[1] using the customary precautions for the protection of the proteins. This procedure resulted in an increase of the $T/E\,{}^{1\%}_{1\,cm.}$ (540 mμ) from an initial 2.34 to 2.62 in one experiment and from 2.40 to 2.54 in a second one.

DISCUSSION

The analytical procedure described is based upon the principle of maintaining the Cu ++ required for the biuret reaction in solution by means of complex-forming ions. This principle has been described and applied by others[2, 3]; however, the reagent here described is somewhat simpler to prepare, and more stable than previously described ones. It results in virtually immediate formation of the biuret complex and yields a somewhat higher $T/E\,^{1\%}_{1\ cm.}$ (540 mμ) than the tartrate reagents. The procedure described has been in use in this laboratory for three years, has been applied to a considerable range of materials, and has been found reliable and reproducible. The reagent blank tends to vary slightly, and should be checked for each fresh reagent; the resultant $T/E\,^{1\%}_{1\ cm.}$ (540 mμ) values for the copper biuret complex are independent of this blank. Some attention has been given to the possibility of using less elaborate instruments for the colorimetry. It is felt that any instrument of the spectrophotometer type is satisfactory, but that filter-colorimeters do not give sufficiently narrow band width to permit accurate determinations. This is in accord with the experience of others.[4]

Data presented here, and later experience with this reagent, do not indicate significant differences between the $T/E\,^{1\%}_{1\ cm.}$ (540 mμ) of albumins and of globulins; plasma proteins of different albumin/globulin ratios show extinction coefficients identical within the limits of experimental error. This is in agreement with findings with other reagents.[5]

The chemistry of the biuret reaction has been discussed in several communications (cf.[6]). While some difference between $T/E\,^{1\%}_{1\ cm.}$ values for egg albumin and plasma proteins was described in,[2] differences in extinction coefficients of "native" plasma versus "low-temperature-low-ionic-strength-solvent" fractions have not been discussed previously. The data presented indicate that an increase in $T/E\,^{1\%}_{1\ cm.}$ (540 mμ) results from treatment of plasma proteins with ethanol at low temperatures at pH 4.8, and that this effect is not caused by removal of "interfering" substances in the process of purification.

The results suggest that some peptide linkages are not available for formation of biuret complex in the native protein, but become available during the isolation procedures. If borne out by more extensive work this conclusion means that a critical application of the biuret reaction should detect early changes in protein structure not readily revealed by other methods now available.

The one factor limiting applicability of the present reagent is the development of turbidity in the reaction mixtures. In practice this is overcome by reading solutions shortly after addition of this reagent, or, if preferred, by storage followed by centrifugations (cf.[7]). The reaction is not related to the presence of copper in the solutions, nor, inherently, to the pro-

tein component. The material precipitating appears to represent, in large measure, lipids, especially phospholipids, previously protein bound and released by the alkali treatment.

SUMMARY

1. A new simplified stable biuret reagent, employing NH_4OH to maintain the required Cu ++ in solution, has been described.

2. Its application to the determination of plasma protein concentrations has been discussed, and the optimal protein concentration range and conditions have been described.

3. The effective extinction coefficient of the biuret complex from "native" plasma proteins was found to be $T/E\,^{1\%}_{1\,cm.}$ (540 mμ) = 2.37 ± 0.04; for various fractions obtained by low temperature-solvent fractionation of such plasmas $T/E\,^{1\%}_{1\,cm.}$ (540 mμ) was found to be larger, 2.82 ± 0.05.

4. The nature of this difference has been discussed on the basis of experimental material, and it is proposed that the biuret reaction reflects slight changes in protein structure resulting from the fractionation procedure.

The authors are indebted to Dr. E. Hayes of the Armour Company, Chicago, Ill., for making available the bovine and canine plasma fractions.

The authors wish to thank Dr. E. J. Cohn, Dept. of Physical Chemistry, Harvard Medical School, Boston, Mass., for making available the human plasma fractions.

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DETERMINATION OF INULIN IN THE PRESENCE OF HIGH GLUCOSE CONCENTRATIONS BY THE GLUCOSE AUTOXIDATION PROCEDURE

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IN A previous report[1] it was shown that the autoxidation of glucose in an alkaline solution may be substituted for the usual yeast fermentation in the determination of plasma inulin concentrations in the presence of a normal glucose plasma concentration. Data reported here show that the procedure is applicable in the presence of high plasma glucose concentrations such as would be encountered in a determination of glucose-Tm in renal function studies.

EXPERIMENTAL

The procedure as previously reported[1] was modified by extending the heating period for glucose autoxidation to fifteen minutes instead of ten minutes. In precipitating the protein, the 4 ml. of 1.0 N NaOH was added slowly with constant shaking to avoid incomplete inulin recoveries. Precautions were taken to insure that the water level in the bath during heating was maintained constantly at 7 cm. above the end of the tube.

In determining the per cent light transmission of the several plasma-inulin solutions with various glucose concentrations, the galvanometer of the Evelyn colorimeter was set at 100 per cent transmission with a plasma blank to which glucose had been added in an amount corresponding to that concentration present in the experimental tubes. The inulin* was recrystallized five times from solution in hot water by the addition of acetone, and it was finally washed with acetone and air dried.

RESULTS

In preliminary experiments, inulin was found to resist destruction when heated for twenty minutes in 0.8 N NaOH. Since the destruction of glucose was incomplete occasionally with a heating period of ten minutes, the period of heating was extended to fifteen minutes.

The results of experiments with the addition of no glucose and the addition of sufficient glucose to give a plasma concentration of 100 mg. per cent and 1,000 mg. per cent will be found in Table I. It will be seen that there is a significant difference between the mean values for light transmission with the addition of no glucose and the addition of 1,000 mg. per cent glucose, when calculated from the t value,[2] with the exception of 5 mg. per cent inulin level; but there is no significant difference in the mean values between 100 mg. per cent and 1,000 mg. per cent glucose.

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*Purchased from Eimer and Amend, New York, N. Y.

TABLE I. THE EFFECT OF GLUCOSE CONCENTRATION ON THE DETERMINATION OF INULIN IN PLASMA AS EXPRESSED BY PER CENT LIGHT TRANSMISSION

INULIN CONCENTRATION (MG. PER CENT)	GLUCOSE ADDED (MG. PER CENT)	NO. OF DETERMINATIONS	MEAN LIGHT TRANSMISSION G (PER CENT)	STANDARD DEVIATION OF G	PROBABILITY OF DIFFERENCES FROM t VALUES
5	None (A)	11	82.6	1.04	A C =
	100 (B)	6	83.4	0.75	>0.05
	1000 (C)	10	83.4	1.60	B C =
					1.0
10	None (A)	11	68.3	1.35	A C =
	100 (B)	6	69.5	0.46	<0.05
	1000 (C)	11	69.8	1.65	B C =
					>0.5
20	None (A)	11	46.2	1.41	A C =
	100 (B)	6	47.4	1.18	<0.05
	1000 (C)	10	48.0	2.03	B C =
					>0.5
30	None (A)	11	31.5	1.00	A C =
	100 (B)	6	31.8	1.08	<0.05
	1000 (C)	11	32.7	1.30	B C =
					>0.15

Since the plasma glucose concentration is known in a determination of glucose-Tm, the preparation of the blank solution containing the appropriate amount of glucose would entail little extra work. The inulin concentration could be read from a graph prepared by the addition to plasma of inulin in varying concentrations and glucose equivalent to 100 mg. per cent, as in the first report.[1]

If it is more convenient to run the inulin determination before the glucose determination, in studying the glucose-Tm, the former may be run against a blank containing 100 mg. per cent glucose. The inulin concentration then may be read from the appropriate standard curve, in a family of curves for various glucose concentrations, prepared in the usual manner against a blank containing 100 mg. per cent glucose added. Both methods have been used in this laboratory.

SUMMARY

The principle of autoxidation of glucose in an alkaline solution with heat has been applied to the determination of plasma inulin concentrations in the presence of a 1 per cent plasma glucose concentration. The period of heating was extended to fifteen minutes to insure complete glucose destruction, and the plasma blank was adjusted in glucose concentration to correspond to the glucose concentration of the unknown.

The author wishes to acknowledge with appreciation the assistance of Miss Alice Cottam and Miss Margaret Hedrick in these experiments.

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THE EFFECT OF MERTHIOLATE ON THE INFECTIVITY OF CERTAIN VIRUSES

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IN THE course of routine diagnostic neutralization tests in mice performed in this laboratory, it was noted that with certain viruses an occasional positive test would be obtained which was not consistent with the findings on other prior and subsequent sera from the same patient. In one such instance it was noted that the specimen giving the positive result smelled of Merthiolate, and this suggested that the addition of this chemical to the serum might interfere with the neutralization test when used to detect antibodies against some viral agents.

Merthiolate has been extensively used as a bacteriostatic agent for sera which must be stored at ordinary icebox temperatures for prolonged periods. Hammon and Reeves[1] reported that the use of this preservative did not interfere with neutralization tests for Western equine encephalomyelitis or St. Louis encephalitis. The purpose of this paper is to show that while Hammon's findings can be confirmed, there are at least three viruses against which false positive neutralization tests result when Merthiolate is added to the serum in a dilution of approximately 1:10,000.

MATERIALS AND METHODS

Virus.—The following strains were used in this study: (a) the McMillan strain of Western equine encephalomyelitis (WEE); (b) the Massachusetts strain of Eastern equine encephalomyelitis (EEE); (c) the Nakayama strain of Japanese encephalitis (Jap); (d) the Florio strain of Colorado tick fever (CTF); (e) the Webster strain of St. Louis encephalitis (SLE); (f) the WE strain of lymphocytic choriomeningitis (LCM); and (g) the Army Medical School strain of encephalomyocarditis (EMC). The virus of LCM has been maintained by serial passage in guinea pigs and the remainder of the agents by serial passages in mice. All of these viruses remain relatively stable when stored in glass ampoules in dry ice.

Preservative.—The Merthiolate was obtained from Eli Lilly Co. It was prepared as a 1 per cent solution, filtered through Seitz EK pads, and stored at 4° C. The final dilutions were made at the time of use.

Neutralization Test.—The technique of the neutralization tests was that routinely used in this laboratory.[2] However, instead of pooled human negative Wassermann serum, the normal serum was obtained from rabbits. To the normal rabbit serum Merthiolate was added in a proportion double that of the desired final concentration. This serum containing Merthiolate was distributed in Wassermann tubes in 0.2 ml. amounts, to which test virus was then added in 0.2 ml. amounts in serial tenfold dilutions. All tests were incubated one hour at

From the Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School.

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37° C. before inoculation. The mice were inoculated by the intracerebral route except in the case of encephalomyocarditis, where the intraperitoneal route was used. Titrations were read daily for periods of ten days to two weeks and 50 per cent end points were determined according to the method of Reed and Muench.[3]

Sera.—With the exception of WEE and the first two LCM titrations, all immune sera were prepared by multiple injections of infectious mouse brain into rabbits. After preliminary titrations of trial bleedings, the rabbits were exsanguinated, using sterile precautions. The resultant serum was stored in small amounts at −20° C. for future use. The immune serum used with WEE was prepared in mice in a similar manner. The earlier LCM titrations were controlled with serum from an individual convalescent from an infection with LCM, from whom the virus was isolated and whose serum was shown to contain both complement-fixing and neutralizing antibodies in rising titer.

TABLE I. EFFECT OF VARIOUS DILUTIONS OF MERTHIOLATE ON THE INFECTIVITY OF VARIOUS VIRUSES

TEST VIRUS	TITER OF VIRUS						
	NO. MERTHIOLATE	DILUTION OF MERTHIOLATE IN SERUM					
		1:1,000	1:4,000	1:5,000	1:10,000	1:20,000	1:40,000
Eastern equine encephalomyelitis	8.7*	8.7	8.0		7.8		
Western equine encephalomyelitis	8.1	8.1	8.1		8.1		
St. Louis encephalitis	6.4		6.0		6.0		
St. Louis encephalitis	7.0	6.5	6.8		6.8		
Japanese encephalitis	6.2	6.5			5.8	6.4	6.3
Lymphocytic choriomeningitis	5.6	1.1			3.3		
Lymphocytic choriomeningitis	5.1	1.8	1.4		3.5		
Lymphocytic choriomeningitis	7.4	4.8	2.8	4.8	4.6	5.5	5.7
Colorado tick fever	5.0	2.0	2.0		2.0	2.5	
Colorado tick fever	6.4			2.6	3.4	3.2	3.2
Encephalomyocarditis	7.5	3.4		3.6	3.8	4.8	5.8
Encephalomyocarditis	7.4	2.9		2.6	2.8	3.8	4.5
Encephalomyocarditis	7.9	2.6		2.7	2.6	3.6	3.5

*Reciprocal of the log of the dilution.

EXPERIMENTAL

The findings summarized in Table I confirm the observations of Hammon[1] that the presence of Merthiolate in dilutions commonly used as a bacteriostatic agent has no effect on the viruses of WEE and SLE. Similarly, the viruses of EEE and Japanese encephalitis are also unaffected. Thus, these agents titrate at approximately the same level in the presence of normal rabbit serum and in the presence of serum containing various dilutions of Merthiolate. A different picture was obtained, however, in the case of LCM, CTF, and EMC. With these three viral agents, Merthiolate in a 1:10,000 dilution so reduces the virus titer that were an unknown serum containing this reagent to be tested by a neutralization test in mice, a false positive test would result. Even when the Merthiolate is diluted as much as 1:40,000, a similar loss of titer occurs with CTF and EMC. At this level the action of the reagent is less marked with LCM but is still sufficient to confuse diagnostic tests.

SUMMARY AND CONCLUSIONS

Seven viral agents have been titrated in mice in the presence of various dilutions of Merthiolate which cover the range commonly used to preserve serums. The infectivity of four of these agents appears to be unaffected by the drug in the dilutions used. Three of the agents, however, are so markedly inhibited as to interfere seriously with in vivo tests. It is likely that as new viruses are adapted to in vivo tests, other strains will be encountered which are susceptible to Merthiolate.

It is concluded that Merthiolate in concentrations sufficient to control bacterial contamination is an unsatisfactory preservative for serums which may be used for neutralization tests in mice against certain viruses.

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THE EFFECT OF TUBERCLE BACILLUS CONCENTRATION PROCEDURES ON FUNGI CAUSING PULMONARY MYCOSES

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SEVERAL pathogenic fungi cause pulmonary disease clinically and roentgenologically indistinguishable from tuberculosis. Although tuberculosis is the commonest cause of chronic lung disease, awareness of the etiologic role played by fungi is increasing. From a laboratory standpoint it would be most desirable if the procedures now in use for the cultivation of the tubercle bacillus were found applicable to the isolation of fungi. The question arises whether pathogenic fungi in gastric washings or sputum could survive the concentration and digestion procedures used for the isolation of *Mycobacterium tuberculosis* and whether surviving fungi could grow on the commonly used tubercle bacillus media. In other words, can the mycotic nature of a pulmonary infection be established in the laboratory even if the initial clinical diagnosis is tuberculosis and no special tests for fungi are performed? In this study the effect of several tubercle bacillus concentration procedures on the survival of a selected group of pathogenic fungi is reported.

MATERIALS AND METHODS

The large quantities of sputum required for these tests were obtained from the bacteriology laboratories of the Georgia Department of Public Health. As the sputum was received, it was pooled and stored in a refrigerator until a sufficient quantity was on hand for testing the ability of a particular fungus to survive the concentration procedures.

Before use, the pooled sputum was homogenized by shaking in a paint conditioner for ten minutes. Following homogenization, the sputum was centrifuged at 2,000 r.p.m. for thirty minutes and the supernatant used in the concentration tests. Centrifugation removed yeasts and yeastlike organisms such as *Candida albicans* and *Geotrichum candidum* which would have interfered with the experiments.

The fungi used were: *Blastomyces dermatitides*—strain No. 87; *Candida albicans*—strain No. 146; *Coccidioides immitis*—strain No. 286; *Cryptococcus neoformans*—strain No. 27; *Geotrichum candidum*—strain No. 151; *Histoplasma capsulatum*—strain No. 105; *Nocardia asteroides*—strain No. 37.* Because of the technical difficulties presented by its anaerobic growth requirements, *Actinomyces bovis*, although a frequent cause of pulmonary infection, was not included in these tests.

In order to permit recovery of the fungus from 40 ml. of sputum prior to treatment, it was found necessary to adjust the size of the inoculum for each fungus species. The form and quantity of the fungus utilized in the inoculum are presented in Table I, where the figures represent the minimum quantities of fungus cells necessary for the isolation of the test fungi from untreated sputum on Sabouraud dextrose agar. With the diphasic organisms the tissue phase rather than the mycelial phase was used as the inoculum, since it is the tissue phase that occurs in sputum.

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*These strains are maintained permanently in this laboratory's culture collection.

TABLE I. TYPE OF INOCULUM AND QUANTITY USED TO SEED SPUTUM

TEST FUNGUS	STOCK CONCENTRATION*	QUANTITY ADDED PER 40 ML. OF SPUTUM
B. dermatitidis Yeast-phase	4.0%	7.0 ml.
C. albicans Yeast-phase	3.0%	1.0 ml.
C. immitis Tissue-phase	Ground caseous lymph nodes from 10 guinea pigs	9.0 ml.
C. Neoformans	4.0%	10.0 ml.
G. candidum Yeast-phase	0.01%	1.0 ml.
H. capsulatum Yeast-phase	4.0%	10.0 ml.
N. asteroides Ground Mycelium	5.0%	2.5 ml.

*Determined in a Hopkins centrifuge tube.

The yeast phase of *B. dermatitidis* was obtained by growing the fungus for five days on a special nutrient medium* at 37° C.

C. albicans, C. neoformans, and *G. candidum* were grown at 37° C. for four days on Sabouraud dextrose agar.

At the present time media are not available for converting the mycelial phase of *C. immitis* to the tissue phase in vitro, but sufficient quantities of spherules and endospores were obtained from infected animals by the following procedure: A suspension of arthrospores of *C. immitis* was injected intraperitoneally into guinea pigs. After four weeks the guinea pigs were autopsied and caseous lymph nodes were excised and ground in a Ten-Broeck tissue grinder. The material thus obtained contained numerous spherules and arthrospores. A predetermined quantity of ground-infected tissue was then added to the sputum used in the concentration studies.

The yeast phase of *H. capsulatum* was obtained by scraping off the five-day growth on Francis cystine glucose agar incubated at 37° C.

Ground mycelium was used as the inoculum for *N. asteroides* as this fungus does not sporulate. The fungus was grown on Sabouraud dextrose agar at 25° C. for a period of one month after which the growth was scraped off, ground, and suspended in physiologic saline.

Four digestion and concentration procedures, representative of the methods in current use by bacteriology laboratories, were selected:

Method I. Sodium Hydroxide with Mechanical Shaking.[1]—Equal volumes of seeded sputum and 4.0 per cent sodium hydroxide (40 ml. each) were mixed in a 250 ml. centrifuge bottle, shaken for ten minutes in a paint conditioning machine, and centrifuged at 2,000 r.p.m. for fifteen minutes. After removal of the supernatant fluid, the sediment was neutralized with 2.0 per cent hydrochloric acid and four tubes of each of four different solid media were inoculated with 0.1 ml. of sediment. The cultures then were incubated at 37° C. and examined periodically for the growth of fungi over a period of six weeks. The same isolation procedure was used for the three concentration methods described below.

Method II. Sodium Hydroxide without Mechanical Shaking[2].—In this method, 80 ml. of 4.0 per cent sodium hydroxide were mixed with 40 ml. of seeded sputum, incubated at 37° C. for twenty minutes and shaken† every five minutes by hand for the first fifteen minutes. After incubation the sputum-hydroxide mixture was centrifuged for fifteen minutes at 2,000 r.p.m. The sediment was then neutralized with 2N hydrochloric acid.

Method III. Sulfuric Acid Procedure[2].—A mixture of 80 ml. of 5.0 per cent sulfuric acid and 40 ml. of seeded sputum was kept at room temperature for ten minutes and shaken

*A nutrient agar used in the medical mycology laboratory of the Duke University Medical School by Dr. Norman F. Conant. Formula:

Peptone	10.0 Gm.	Beef extract	3.0 Gm.
Dextrose	10.0 Gm.	Agar	20.0 Gm.
Sodium chloride	5.0 Gm.	Distilled water	980.0 ml.

†Arbitrarily fixed at twenty swirls.

by hand* at the end of the first five minutes. At the end of ten minutes, 40 ml. of sterile physiologic saline was added to the above mixture. The diluted sputum then was spun at 2,000 r.p.m. for fifteen minutes. The sediment thus obtained was neutralized with 4.0 per cent sodium hydroxide before inoculation into the media tubes.

Method IV. Trisodium Phosphate[3].—Forty milliliters each of 10.0 per cent trisodium phosphate and seeded sputum were mixed in a centrifuge tube and allowed to digest at room temperature until the sputum became clear (approximately four hours). The cleared sputum was centrifuged for fifteen minutes at 2,000 r.p.m. and the sediment neutralized with 2N hydrochloric acid before being inoculated into the media tubes.

The concentration tests were run in duplicate with all of the fungi except *C. neoformans*. Tests on this fungus were run in quadruplicate owing to the erratic preliminary results obtained.

The four media used in culturing the various sediments were:

a. Lowenstein-Jensen medium[2]
b. Petragnani's medium[4]
c. American Trudeau Society medium[5]
d. Sabouraud dextrose agar (Difco)

The first three are those commonly used for the isolation of the tubercle bacillus.

Sabouraud dextrose agar, the medium most widely used for the isolation of pathogenic fungi, served as a control.

Adequacy of the concentration procedures was shown by the fact that tubercle bacilli which were present in all of the batches of pooled sputum were isolated consistently in every run.

RESULTS

The ability of the test fungi to survive the concentration procedures was influenced markedly by the technique employed. The average rate of recovery with all four methods was approximately 25 per cent, as revealed in Table II.

TABLE II. OVER-ALL EFFECT OF CONCENTRATION PROCEDURES ON THE TEST FUNGI

CONCENTRATION PROCEDURE*	TOTAL NO. TUBES INOCULATED	NO. POSITIVE CULTURES	PER CENT POSITIVE
I	256	78	30.5
II	256	15	5.9
III	256	51	19.9
IV	256	110	43.0
Total	1024	254	24.8

*I Sodium hydroxide with mechanical shaking.
II Sodium hydroxide without mechanical shaking.
III Sulfuric acid.
IV Trisodium phosphate.

The individual fungus species differed considerably in their ability to survive particular concentration procedures (Table III). Recovery, depending on species and concentration procedure, varied from 0 to 100 per cent. The poorest recoveries resulted from the use of NaOH as the digestant (procedures I and II). Of the seven fungus species tested, only *C. neoformans* and *N. asteroides* survived procedure II. Even when the amount of NaOH was halved, as in procedure I, these species again were the only survivors. The destructive action of this alkali was not due to pH alone, since the pHs in procedures I, II and IV were 11.6, 11.5 and 11.1, respectively.

In general, as shown in Table IV, no significant difference existed among the isolation media with respect to the number of positive cultures obtained,

TABLE III. EFFECT OF TUBERCLE BACILLUS CONCENTRATION PROCEDURES ON SPECIFIC PATHOGENIC FUNGI

FUNGUS SPECIES TESTED	NUMBER OF TU. ES INOCULATED PER PROCEDURE IN DUPLICATE RUNS	CONCENTRATION PROCEDURE*							
		I		II		III		IV	
		POSITIVE		POSITIVE		POSITIVE		POSITIVE	
		NO.	%	NO.	%	NO.	%	NO.	%
B. dermatitidis	32	0	0	0	0	1	3.1	8	25.0
C. albicans	32	0	0	0	0	32	100.0	28	87.5
C. immitis	32	0	0	0	0	4	12.5	8	25.0
C. neoformans	64†	57	79.7	1	1.6	14	21.9	29	45.3
G. candidum	32	0	0	0	0	0	0	1	3.1
H. capsulatum	32	0	0	0	0	0	0	4	12.5
N. asteroides	32	27	84.4	14	43.8	0	0	32	100.0

*I Sodium hydroxide with mechanical shaking.
II Sodium hydroxide without mechanical shaking.
III Sulfuric acid.
IV Trisodium phosphate.
†Quadruplicate runs.

although, following procedure IV, a twofold greater number of fungus cultures developed on Sabouraud agar than on any of the other media. No ready explanation can be given to account for the apparent superiority of this medium over the others for, in preliminary tests with untreated fungi, it was found that all of the fungi grew equally well on the tubercle bacillus media and Sabouraud agar at 37° C.

From the data in Table III it appeared that four of the fungi, *B. dermatitidis, C. immitis, G. candidum,* and *H. capsulatum,* were adversely affected by all of the concentration methods tested, for none had a recovery rate higher than 25 per cent.

Of the remaining fungi, *C. albicans* was distinguished by its complete destruction by NaOH and its excellent resistance to H_2SO_4. *N. asteroides,* conversely, could not withstand the acid but was significantly resistant to NaOH. Both of these fungi showed excellent resistance to Na_3PO_4. *C. neoformans* tolerated NaOH well, but only at the comparatively low concentration used in procedure I.

Other workers have noted that the concentration methods used for *M. tuberculosis* kill pathogenic fungi. Benbow and associates,[6] Hager and co-workers,[7] and Kirby and McNaught[8] failed to isolate *N. asteroides* from body fluids concentrated with NaOH. Although in our experiments a recovery of 84.4 per cent was obtained with *N. asteroides* following procedure I, it must be pointed out that Benbow and co-workers,[6] Hager and associates,[7] and Kirby and McNaught[8] probably used a more severe NaOH procedure similar to our procedure II, and that the amount of fungus present in the body fluids concentrated by them was smaller than in our test sputum.

Cox and Tolhurst[10] found that sputum from a patient with cryptococcosis failed to infect guinea pigs after concentration with NaOH.

Smith[9] reported that *C. immitis* was killed by the concentration methods used for *M. tuberculosis.* In the present study, recoveries of 12.5 per cent and 25.0 per cent were obtained following concentration with H_2SO_4 and Na_3PO_4: there was no recovery with NaOH.

TABLE IV. INFLUENCE OF MEDIA ON THE ISOLATION OF FUNGI FOLLOWING CONCENTRATION

FUNGUS SPECIES TESTED	NUMBER TUBES OF EACH MEDIUM INOCULATED IN DUPLICATE RUNS PER PROCEDURE	CONCENTRATION PROCEDURE*															
		I				II				III				IV			
		MEDIA†															
		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
B. dermatitidis	8	0	0	0	0	0	0	0	0	0	0	0	1	8	0	0	0
C. albicans	8	0	0	0	0	0	0	0	0	8	8	8	8	8	4	8	8
C. immitis	8	0	0	0	0	0	0	0	0	0	1	3	0	3	3	2	0
C. neoformans	16‡	16	13	12	10	0	0	1	0	4	4	4	2	11	7	4	7
G. candidum	8	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
H. capsulatum	8	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0
N. asteroides	8	3	8	8	8	3	4	4	3	0	0	0	0	8	8	8	8
Total Number Cultures	64	19	21	20	18	3	4	5	3	12	13	15	11	43	22	22	23
% Positive culture per type of medium		29.7	32.9	31.3	28.1	4.7	6.3	7.8	4.7	18.8	20.3	23.4	17.2	67.2	34.4	34.4	35.9

*I Sodium hydroxide with mechanical shaking.
II Sodium hydroxide without mechanical shaking.
III Sulfuric acid.
IV Trisodium phosphate.

†A, Sabourand dextrose agar.
B, Lowenstein-Jensen.
C, Petragnani.
D, Trudeau Society.

‡Quadruplicate runs.

Although in general the conditions prevailing during concentration are of such nature that few pathogenic fungi survive them, it is apparent that some can be isolated occasionally. It is therefore important that laboratory workers do not discard any fungus that develops in culture tubes following digestion without prior examination for possible diagnostic importance.

It is clear that the routine examination of sputum or body fluid for the presence of viable fungi must depend upon the use of untreated specimens utilizing the standard techniques for the isolation of fungi.

SUMMARY AND CONCLUSIONS

The effects of four different tubercle bacillus concentration procedures were tested on seven pathogenic fungi.

It was found that, in general, most pathogenic fungi cannot survive these procedures.

Nocardia asteroides was isolated from sputum in every test using trisodium phosphate as the digestant.

Candida albicans was recovered in every case with the sulfuric acid procedure.

Cryptococcus neoformans survived digestion with NaOH in the procedure utilizing a 1 to 1 ratio of sputum and 4.0 per cent alkali.

The digestion procedures commonly used in the isolation of tubercle bacilli cannot be relied upon for the isolation of fungi from sputum. Nevertheless, any fungi that develop on the isolation media should be examined carefully for possible diagnostic importance.

We wish to express our thanks to Dr. S. E. Miller, Medical Director, Chief, Division of Industrial Hygiene, U. S. Public Health Service, Washington, D. C., for suggesting and encouraging this investigation.

We wish to take this opportunity to thank Dr. D. J. Sunkes, Director of Laboratories, for his assistance in obtaining sputum.

We also wish to thank Dr. Martin Cummings, Chief, Tuberculosis Section, Lawson Veterans Administration Hospital, Chamblee, Ga., for his technical advice and helpful criticisms.

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THE RE-USE OF RABBITS FOR TESTING VIRULENCE OF CORYNEBACTERIUM DIPHTHERIAE

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THE method devised by Fraser and Weld for determining the virulence of *Corynebacterium diphtheriae* by intracutaneous injection into rabbits has been widely used since it was first introduced in 1926.[1] The use of one rabbit for both test and control generally yields entirely satisfactory results, and the method has the great advantage of economy when large numbers of cultures are to be tested. However, when only a few cultures are tested on one rabbit the procedure is expensive. The question has frequently arisen, therefore, as to whether rabbits, once used for the test, could or could not safely be re-used for virulence testing at a later date. Re-use of such rabbits is known to be the practice in some laboratories, yet we have not been able to find any experimental data on which to base a definite statement concerning it. Because many rabbits previously employed for virulence tests were available in this laboratory, it was decided to assay their value for re-use in testing the virulence of *C. diphtheriae*.

EXPERIMENTAL

The standard Fraser-Weld method for testing virulence, as described by the American Public Health Association,[2] was used for all rabbits. Injections were made with forty-eight to seventy-two hour heart-infusion broth cultures. The majority of rabbits were normal when first used for routine virulence tests. This first completed virulence test is referred to in this paper as the "diagnostic injection." A few of the rabbits had been used for serum production (other than diphtheria) when they received their diagnostic injections.

A total of thirty-one rabbits were used in this study. Four of these, serving as "controls," were each given, as a diagnostic injection, the following four known strains of *C. diphtheriae:* (1) gravis-virulent; (2) mitis-virulent; (3) minimus-virulent; and (4) mitis-avirulent, on the same date. The second or "re-use injections" were given after intervals of seven days to one month. The other twenty-seven rabbits used in this study had been subjected to routine virulence testing with cultures of unknown virulence and were, therefore, typical of the animals re-used in most laboratories. The number of positive reactions on each rabbit had varied from one to twenty-one, the most common numbers being from twelve to seventeen. The intervals between diagnostic and re-use injections varied from seven days to seven months. Twenty-one of the rabbits were retested after an interval of six to seven months. The re-use injections for all rabbits consisted of the following known strains of *C. diphtheriae:* (1) gravis-virulent; (2) mitis-virulent; (3) minimus-virulent (for the last twenty-one rabbits a second minimus culture of weaker virulence was also included); and (4) mitis-avirulent.

RESULTS

Table I shows, in condensed form, the results obtained from the reinjection of thirty-one rabbits, after varying time intervals. Only five of these rabbits gave entirely typical reactions after reinjection. Twelve other rabbits showed

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TABLE I. REACTIONS OF RABBITS, ONCE USED FOR TESTS OF VIRULENCE OF C. DIPHTHERIAE, TO RE-USE INJECTIONS

INTERVALS BETWEEN DIAGNOSTIC AND RE-USE INJECTIONS	POSITIVE REACTIONS IN PREVIOUS DIAGNOSTIC TESTS	TYPES OF REACTIONS TO RE-USE INJECTIONS					TOTAL RABBITS
		TYPICAL*	MARKED EDEMA AND INCREASED REACTIONS*	DECREASED AND ATYPICAL REACTIONS WITH NECROSIS	NO REACTION OR VERY SLIGHT	MITIS AND GRAVIS TYPICAL*; MINIMUS OR AVIRULENT, OR BOTH, ATYPICAL	
7 da.-14 da.	2-3		1		2		3
14 da.-1 mo.	1-9	3	1				4
1 mo.-4 mo.	1-4		1	1	1		3
6 mo.-7 mo.	3-21	2	9	1	3	6	21
Totals		5	12	2	6	6	31

*Readily recognizable as positive reactions to virulent strains of *C. diphtheriae*.

very marked edema with unusually large and deep necrotic areas at the sites of injection of virulent strains. Such reactions would be recorded as "positive" in routine virulence tests. They probably represent reactions enhanced by allergy as described by Frobisher, Parsons, and Updyke.[3-5] Thus, seventeen rabbits (55 per cent) gave definitely positive reactions to virulent *C. diphtheriae*.

Six of the remaining rabbits gave positive reactions with virulent mitis and gravis strains, but abnormal reactions with either the minimus or avirulent strains, or both. Six other rabbits gave no reactions, or very slight reactions, and the last two gave decreased and very atypical reactions. A total of fourteen (45 per cent) thus failed to react in such a manner that definite and reliable determinations of virulence could be made.

The intervals between injections, and the numbers of positive reactions resulting from the diagnostic injections, seemed to play no role in determining the types of reactions to the re-use injections. We did not test the blood of these rabbits for the presence of antitoxin before making the re-use injections.

DISCUSSION

In our opinion, the data here presented would indicate that the re-use of rabbits for virulence testing is a definitely questionable procedure. However, our experience has shown that rabbits previously used for diphtheria virulence tests may be used later as a source of blood for culture media, including media for *C. diphtheriae*, or for other types of test injections. Whenever possible, it has been our practice to let the lesions on the back heal before subjecting the rabbit to bleeding.

SUMMARY

Thirty-one rabbits used for virulence testing of *C. diphtheriae* according to the Fraser-Weld method were retested by this same method after intervals of from seven days to seven months.

The data here presented indicate that repeated use of rabbits for testing virulence of *C. diphtheriae* results frequently in deceptive or incorrect results.

Rabbits once used for testing virulence of *C. diphtheriae* may be used as a source of blood for culture media or for other experimental procedures, antibody production, or for other types of test injections.

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AN IMPROVED TRAP-TRANSPORT CAGE FOR MONKEYS

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AN IMPROVED trap-transport cage has been designed to aid investigators confronted with the problem of catching and restraining monkeys.

The new trap cage has three principal advantages over others now commonly in use: (1) It can be adapted to fit any monkey cage which opens by means of a vertically sliding service door. (2) It can be attached to the main cage, leaving both hands of the operator free, and making it unnecessary to hold the trap until the monkey enters. (3)A net can be placed inside the trap before the monkey enters. The animal may then easily be removed without danger to the operator, and with a minimum of emotional upset to the monkey.

Other workers[1] have found the use of a net in conjunction with a trap cage effective in the handling of monkeys. Their cage, however, must be lifted off the net in order to remove the monkey, and more than one operator usually is required to effect the removal. In addition, there is no provision for attaching the trap to the main cage.

The transport cage is 10 in. wide by 12 in. high by 15 in. deep, as shown in Fig. 1. There is a vertically sliding door in front, with a lock to hold the door in the open or closed position. A woven, linen net, with a drawstring arrangement, is made to fit neatly inside the trap, secured in place with removable steel suspension rods which fit through grommets in the net. With the net open, the drawstring is anchored along the side of the trap cage.

In using the trap, the service door of the main cage is lifted slightly, the lower front projection of the transport cage (a) is inserted over the lower sill of the monkey cage opening. The service door of the main cage is then lifted until it clears the right angle clip (b, b') at the top of the transport cage. The trap is pushed back against the monkey cage and the service door is dropped into place in front of the clip, locking the trap cage in position. The transport cage door (c) is locked in the open position and the monkey is lured into the trap. The lock (d) is a simple sliding latch which fits through small holes in the top and bottom of the door (e, e'). When the animal is in the transport cage the trap door is locked in the closed position. Raising the service door will then release the trap from its fixed position, and it can easily be transported to a work table or laboratory. To remove the monkey from the trap, the suspension rods (f, f', f'') are pulled out, the drawstring is released from its anchored position (g) and pulled taut, the trap-cage door is removed, and the monkey is taken from the trap, held securely in the net.

From the Department of Physiology Northwestern University Medical School.
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Injections may be carried out with the monkey in the net; or, if necessary, it is a simple matter to remove the animal from the net. The entire trapping procedure may be carried out by a single operator, with a minimum of effort, and a maximum of safety and efficiency.

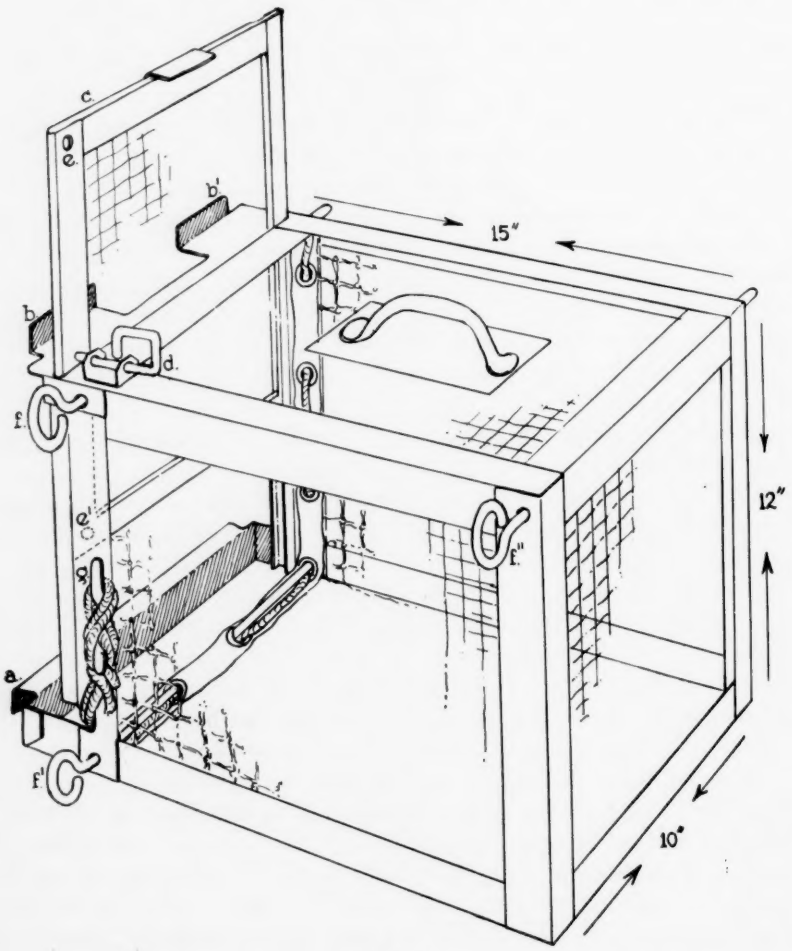

Fig. 1.

Acknowledgment is made to the Acme Sheet Metal Works, Chicago, Illinois, for assistance in the design and construction of this cage.

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